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ROLE OF CALCIUM, ENERGY SUBSTRATES AND NITRIC OXIDE IN MYOCARDIAL DEPRESSION *IN VITRO*

by

KASSIM D. ABOU-CHEHADE

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

Department of Pharmacology

Edmonton, Alberta

Spring, 1996

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ABSTRACT

Isolated cardiac muscle preparations have limited stability *in vitro* and are viable for only a few hours when determining mechanical performance. Factors that may play a role in altering the mechanical stability of these *in vitro* preparations are temperature, energy fuel source, tissue oxygenation, extracellular calcium concentration and workload. Bacterial endotoxin or pro-inflammatory cytokines are known to play a key role in altering mechanical contractile function of cardiac muscle, both *in vivo* and *in vitro*. Recently, it was shown that the exposure of isolated working rat hearts to endotoxin and/or pro-inflammatory cytokines depresses cardiac mechanical function due to the expression of inducible NO synthase (iNOS) activity in the heart.

In this work the effects of energy substrate, extracellular Ca^{2+} concentration, and the protein synthesis inhibitor, cycloheximide (Cx) on cardiac mechanical function and expression of iNOS were investigated. The response over time to cumulative doses (10^{-12}-10^{-7}M) of a β-adrenergic agonist, isoproterenol, was also studied in hearts perfused under different buffer conditions. Isolated working rat hearts paced at 300 beats/min were perfused with recirculating Krebs-Henseleit buffer (>1ng/ml endotoxin) containing either: a) 1.25mM Ca^{2+}/5mM pyruvate, b) 1.25mM Ca^{2+}/0.8mM palmitate in the presence or absence of 10μM Cx or, c) 2.5mM Ca^{2+}/0.8mM palmitate. Cardiac work (cardiac output × peak systolic pressure) was measured as an index of cardiac function. At 1.25mM Ca^{2+}, 2hr perfusion with either palmitate or pyruvate did not significantly alter the loss of function expressed as cardiac work (47.7±8.2%, n=9 vs. 24.7±14.2%, n=5, of initial values respectively). Doubling of buffer Ca^{2+} in palmitate-perfused hearts reduced the loss of cardiac work after

2hr of perfusion, measured at preloads of 13.5 and 15.5 mmHg (n=9, $p<0.05$).

The loss of cardiac work after 2hr perfusion with 1.25mM Ca^{2+}/0.8mM palmitate was abolished by Cx (n=6, $p<0.01$). Cx prevented the increase in iNOS activity seen after 2hr perfusion in ventricular homogenates of 1.25mM Ca^{2+}/0.8mM palmitate-perfused hearts (0.05 ± 0.04 vs. 0.60 ± 0.09 pmol/min/mg protein, $p<0.001$). Cx also prevented the expression of iNOS protein in these hearts as shown by Western blot analysis.

The change in cardiac work in response to the β-adrenergic agonist, isoproterenol (10^{-12}M to 10^{-7}M) was studied in working hearts. A cumulative concentration-response relationship was obtained in separate series of hearts either at the beginning of perfusion (t=0hr) or after 2hr of perfusion. At t=2hr the 1.25mM Ca^{2+}/0.8 mM palmitate group showed a significant decrease ($p<0.05$) in response to isoproterenol at all concentrations compared to that seen at the start of perfusion. Hearts perfused with buffer containing 0.8mM palmitate and either 1.25mM or 2.5mM Ca^{2+} produced similar maximum responses to isoproterenol at a concentration of 3nM and showed no difference in the potency of isoproterenol (EC_{50}, 0.35 ± 0.05 vs. 0.21 ± 0.08 nM respectively, $p>0.05$, n=9) as tested after 2hr of *in vitro* perfusion. Addition of 10μM Cx to 1.25mM Ca^{2+}/0.8 mM palmitate buffer did not show a significant difference in the maximum response at t=2hr of perfusion which was obtained with 1nM isoproterenol compared to the control group. Estimation of the mean effective concentration of isoproterenol at 50% of maximum response (EC_{50}) did not show any significant difference among all groups studied. Therefore, the difference in the maximal response to isoproterenol could be due to alterations in the contractile machinery in cardiac myocytes. Isoproterenol was able to correct the loss of cardiac mechanical function seen after 2hr perfusion at concentrations that stimulated maximal responses in Ca^{2+}/0.8 mM palmitate perfused hearts at t=2hr.

It can be concluded that the loss of cardiac function seen over 2hr of *in vitro* perfusion of the isolated rat heart: a) cannot be explained by the absence of fatty acid, the major energy substrate in the heart, b) is reduced by increasing extracellular Ca^{2+} concentration, c) is abolished by Cx which prevents an increase in iNOS activity and expression, and d) is also shown as a defect in the efficacy but not potency of the positive inotropic action of the β-adrenergic agonist isoproterenol.

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TO THE MEMORY OF MY FATHER FOR HIS CONTINUOUS
ENCOURAGEMNET DURING HIS LIFE,
TO MY BROTHERS AND SISTERS FOR THEIR CARE, SUPPORT AND
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TO MY COUSIN SINE FOR HIS CARE AND SUPPORT

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ABBREVIATIONS

cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate
BH_4	(6R,S)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride
BSA	bovine serum albumin
Cx	cycloheximide
DDW	deionized distilled water
EDTA	ethylene diamine tetra acetic acid
EGTA	ethylene glutamine tetra acetic acid
FAD	flavine adenine dinucleotide
HRP	horse raddish peroxidase enzyme
cGMP	cyclic guanosine monophosphate
IgG-HRP	Immunoglobulin G antibody conjugate to HRP
kb	kilobases
kDa	kiloDalton
LPS	lipopolysaccharide
bNOS	brain or neuronal nitric oxide synthase
cNOS	constitutive nitric oxide synthase
iNOS	inducible nitric oxide synthase
NOS	nitric oxide synthase
NADPH	nicotinamide adenosine diphosphate (reduced form)
L-NA	nitro-L-arginine
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine

PBS	phosphate buffered solution
mRNA	messenger ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
VSM	vascular smooth muscle

CHAPTER I

INTRODUCTION

Nitric oxide (NO) is a ubiquitous, naturally occurring molecule found in a variety of cell types and organ systems. This inorganic molecule is highly lipophilic and synthesized by a family of enzymes known collectively as the NO synthases (NOS) which are widely distributed in mammalian tissues (Knowles and Moncada, 1994; Fostermann *et al.*, 1993; Moncada *et al.*, 1991). These NOS contribute to specific biological functions within the cells (Salter *et al.*, 1991). In the cardiovascular system, NO is a major determinant of basal vascular tone (Kelm and Schrader, 1990), prevents platelet activation, limits leukocyte adhesion to vascular endothelium, and regulates myocardial contractility (Smith *et al.*, 1991; Moncada *et al.*, 1991; Shah and Lewis, 1993; Paulus *et al.*, 1994; Loscalzo and Welch, 1995). Recent investigations indicate that changes in NO production or its degradation may play a role in the pathogenesis of commonly known cardiovascular disorders such as hypotension accompanying septic shock states, essential hypertension, cardiomyopathies, and atherosclerosis (Loscalzo and Welch, 1995). Thus, NO plays both physiological and pathophysiological roles in the cardiovascular system and makes this molecule a major focus for current investigations. More recently, NO has been found to act in the control of cardiac muscle contractile function, both as a negative inotrope (Brady *et al.*, 1993) and chronotrope (Roberts *et al.*, 1992).

In vitro cardiac muscle preparations provide an important tool in understanding the physiological and pathophysiological roles of NO in regulating myocardial contractility. However, these *in vitro* preparations are known to be viable for only a few hours. Factors that play a role in the viability of these preparations are temperature, energy fuel source, extracellular calcium concentration, oxygen supply and workload. For example, the isolated working rat heart loses about 20% of its initial cardiac output by 2hr and 60% by 2.5hr (Taegtmeyer *et al.*, 1980). Recently, it has been shown that endotoxin and/or pro-

inflammatory cytokines present in the perfusate of isolated heart preparations depress cardiac mechanical function due to the expression of inducible NOS (iNOS) activity in the heart (Schulz *et al.*, 1995).

A. LITERATURE BACKGROUND

1. Mechanical properties of heart function:

Heart function, in its narrowest sense, refers to the ability of the heart to pump blood around the circulation and supply oxygen and nutrients to all organs of the organism including the heart muscle itself. One of the best measures of this function is cardiac output corrected for factors such as preload, afterload, heart rate and autonomic tone, all of which influence output. However, in a broader sense heart function can be evaluated in a number of different ways that relate indirectly to the measurement of cardiac output. Thus heart function can be assessed in terms of mechanical function of the whole or components of the heart, heart rate, electrophysiological behaviour (such as electrocardiography and membrane potentials), coronary flow, along with biochemical and histological markers. Such studies can be performed *in vitro* using cardiac tissues (i.e. atria, ventricles, papillary muscles) or intact hearts as well as *in vivo* in the intact animal.

One of the principal features of cardiac muscle mechanics is the relation between the contractile properties of the whole heart and that of the myofilaments. In this respect, force-length relations were extensively studied in cardiac muscle preparations and led to the proposal that the mechanical force, developed by the myofilaments of the heart, is dependent on the initial heart volume (left ventricular end-diastolic volume, or preload) and the developed resistance to the arterial outflow (afterload). Increase in preload leads to an increase in left ventricular volume and, therefore, increase in the ejection capacity

of the left ventricle as shown in pressure-volume studies of the left ventricle (Ross, 1974; Gilbert and Glantz, 1989; Katz, 1990; Brady, 1995). On the other hand, an increase in afterload causes a decrease in muscle fiber shortening and a decrease in contraction capacity of the left ventricle, considering that preload is constant (Quinones *et al.*, 1976; Ross, 1976). These properties which characterize cardiac mechanical function were previously described by Frank and Starling and are now known as Frank-Starling mechanism of cardiac contraction (Starling, 1918; Frank, 1958).

Calcium plays a key role in regulating cardiac mechanical function. The interaction between myosin and actin filaments (known as contractile proteins) of cardiac cells is tightly controlled by the concentration of free calcium ion in the cytosol (Opie, 1995). Because of the very high turnover rate of ATP in the myocardium, a corresponding high rate of mitochondrial production of ATP is required. In this sense, energy fuel source is also another important factor that plays a role in maintenance of cardiac mechanical function. Potential fuel sources for the biosynthesis of ATP in the heart are glucose, pyruvate, lactate, glycogen, fatty acids, and triglycerides (Lopaschuk *et al.*, 1990; Taegtmeyer, 1994). In 1914, Evans suggested that only one third of the heart's energy requirement is supplied by carbohydrates. Cruickshank and his coworkers (1941) suggested that fatty acids account for the rest of the energy requirements of the heart. High levels of free fatty acids inhibit the metabolism of glucose at several sites and are the dominant fuel source for the human heart, especially during the fasted state (Randle, 1976). Inhibition of glucose metabolism by free fatty acids must involve β-oxidation and interfere with glucose utilization, both glycolytic flux and glucose oxidation (Lopaschuk *et al.*,1990). On the other hand, triglycerides are not normally a major energy source for heart muscle after a high lipid meal, yet in

the isolated working rat heart triglycerides can provide about 11% of the energy requirement of the working rat heart (Saddik and Lopaschuk, 1991).

The mechanical performance of the heart *in vivo* can be assessed in terms of cardiac output, using flow probes or more advanced Doppler ultrasound methods (Grayburn *et al.*, 1992), ventricular pressure variations with time (dP/dt), as well as movements of the chamber walls measured using nuclear magnetic resonance spectroscopy. Thus the mechanics of cardiac function depends highly on the characteristics of its contractility, a measure of the rate of contractile force development (Brady, 1991). Contractility reflects the inotropic state of the heart (Opie, 1995). Contractility is a mechanical quality of cardiac muscle that determines performance independent of loading conditions (Parmley, 1979). Contractility should not be influenced by the size or shape of the left ventricle (Opie, 1995). It is agreed in the literature that contractility should be measured in the absence of any changes in workload (preload and afterload), or heart rate. These conditions are known to alter the contractile pattern of the heart (Opie, 1995). Contractility is an important regulator of myocardial oxygen uptake. Factors that increase contractility include digitalis, other inotropic agents, and adrenergic stimulation. In isolated heart perfusions, contractility also rises with increases in heart rate (Opie, 1995). On the other hand, there are situations when contractility decreases, such as during energy depletion by anoxia or ischemia, lowering cytosolic calcium by β-adrenergic blocking agents, mechanical damage of the myocardium by ischemic fibrosis, and in congestive heart failure associated with abnormalities in the control of cytosolic calcium levels (Opie, 1995).

2. The *in vitro* working heart as a model to study heart function :

For many years, cardiac tissues have been isolated and used to study myocardial function. Preparations of ventricular tissues, as well as whole ventricles, have been successfully perfused and studied for assessment of mechanical function and for electrophysiological studies (Carmeleit and Zaman, 1979; Grupp and Grupp, 1984). The most physiologically relevant isolated cardiac tissue preparation is the isolated perfused whole heart. Oscar Langendorff (Langendorff, 1895) deserves credit for first devising a method to allow investigation of the mechanical activity of the completely isolated mammalian heart. This preparation has been used extensively by many investigators (Broadley, 1979; Levi and Mullane, 1990) and is especially useful for investigating the hemodynamics of the coronary vasculature. Perhaps most "physiological" are isolated hearts used in heart/lung preparations where blood is used as the perfusion medium (Hilton and Eichholtz, 1925; Rand *et al.*, 1979). Difficulties with the heart/lung preparation led to the widespread use of isolated buffer perfused heart preparations, the most common being the Langendorff heart (1895) which receives the perfusate in a retrograde manner through the aorta. This was later improved into the working heart by Neely *et al.* (1967) in which the heart is made to perform external work by pumping the perfusate against a hydrostatic pressure head. Crystalloid perfusion solutions include standard Ringer's (Ringer, 1883), solutions such as Locke's (1901) or Krebs'-Henseleit (1932) solutions modified in some studies by the addition of EDTA (Neely *et al.*, 1967; MacConaill, 1985; Taegtmeyer *et al.*, 1980). These crystalloid perfusates have been further modified by adding platelets (Purchase *et al.*, 1986) or red blood cells (Benzi and Lerch, 1992)

The isolated working heart can be used for a large number of electrical, mechanical, and biochemical studies. The function of the isolated heart is usually

assessed in terms of cardiac output (working preparation) or pressure development in the left ventricle (Langendorff preparation and working hearts). The advantage of the isolated working heart model is that heart rate can be controlled and studies can be performed in which preload, afterload, stroke volume, oxygen consumption, and energy substrate utilization can all be measured (Scheuer, 1977). In addition to the facility of studying drug responses, isolated perfused hearts have been used to determine cardiac functional integrity in the setting of various pathological states. For example, hearts that have been taken from diabetic, hypertensive or immunologically challenged animals (i.e. endotoxin challenge) may be perfused and their function evaluated in terms of isovolumic contraction and the Frank-Starling mechanism of heart response (Doring and Dehnert, 1988). The main disadvantages seen in isolated working heart preparations are the lack of neurohumoral and peripheral vascular factors that may affect cardiac function and the fact that they provide descriptions of cardiac function under non-physiological circumstances (Scheuer, 1977). The stability of isolated heart preparations studied over many hours is another problem. Cardiac output falls by about 20% after 2 hr of perfusion in isolated working heart preparations and by more than 60% after 2.5 hr (Taegtmeyer *et al*, 1980).

3. Nitric oxide formation in the heart :

In 1980, Furchgott and Zawadzki demonstrated that acetylcholine-induced relaxation of isolated blood vessels required the presence of an intact endothelium, and that this relaxation was mediated by a diffusible substance that they named endothelium-derived relaxing factor, or EDRF. However, it was not until 1987 that the biological properties of EDRF were shown to be a consequence of the release of NO from endothelial cells (Palmer *et al.*, 1987;

Ignarro *et al.*, 1987). NO is synthesized by an enzyme called NOS (Fig. 1.1). In addition to its production in the vascular endothelium in the heart, NO has been shown to be synthesized by a Ca^{2+}-dependent enzyme in endocardial endothelial cells (Schulz *et al.*, 1991) as well as in cardiac myocytes (Schulz *et al.*, 1992).

a. NO synthases (NOS) - isoforms and structure :

The synthesis of NO is dependent on the activity of at least three distinct NO synthases that catalyze the conversion of L-arginine to NO and L-citrulline, and which requires NADPH, FAD, FMN, (6R,S)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride (BH_4) and calmodulin as co-factors (Fig. 1.1). They are unique in their resemblance to cytochrome P_{450} and exist as calmodulin-dependent or calmodulin-containing isoforms. The three known isoforms of NOS and their characteristics are shown in Table 1.1. Isoforms I (bNOS) and III (eNOS) of NOS are activated by elevation of intracellular Ca^{2+} concentration within the physiological range and are sometimes referred to as cNOS or Ca^{2+}-dependent NOS. Isoform III, or eNOS, can be myristoylated at the N-terminal glycine, a phenomenon which is likely to be responsible for its association to the membrane fraction (Sessa, 1993). Isoform II, or iNOS is not normally present in cells but its expression is induced in response to bacterial endotoxin and/or inflammatory cytokines (Moncada *et al.*, 1991). This isoform of NOS is similar to the other NO synthases in that it also contains a calmodulin regulation sequence. Calmodulin is a tightly bound subunit that copurifies with this enzyme and obviates its requirement for calcium to stimulate NO formation (Cho *et al.*, 1992). The normal heart possesses only Ca^{2+}-dependent NOS activity whereas both cNOS and iNOS were first detected in homogenates of hearts removed from rats challenged with bacterial endotoxin (Schulz *et al.*, 1992). In these rats the activity of iNOS

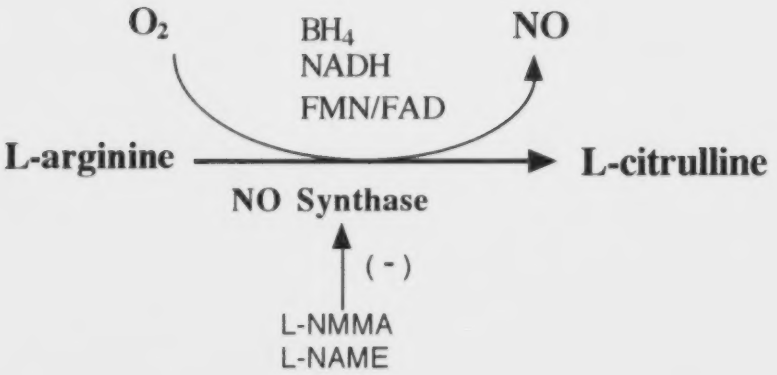


Figure 1.1 Biosynthesis of nitric oxide (NO) and its co-product L-citrulline from L-arginine and molecular oxygen by NO synthase. These synthases exist as dimers and are calmodulin-dependent or calmodulin containing, and bind to (6R,S)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride (BH_4), NADPH, FAD, and FMN to catalyze the unusual five electron oxidation of L-arginine to NO. Analogues of L-arginine, L-NMMA and L-NAME, are specific inhibitors of all isoforms of NO synthase, however, they do not show selectivity between the isoforms.

Table 1.1 Characteristics and tissue distribution of NOS isoforms. Three main isoforms of NOS proteins have been isolated and cloned from various tissues. Isoforms I and III are characterized as constitutive isoforms of NOS that are calcium and calmodulin dependent. They are responsible for the physiological low output release of NO in tissues. Isoform II, or iNOS, is synthesized *de novo* upon stimulation of cells or tissues with bacterial endotoxin and/or pro-inflammatory cytokines and is responsible for the high output release of NO during macrophage activation and in certain disease states. Isoform II is calcium independent although it is tightly bound to calmodulin. Both type I and III NOS exist as dimers and are located in the cytosolic fraction whereas in endothelial cells. Type III NOS is bound primarily to cell membranes as a particulate isoform.

Isoform (Type)	**Subcellular Location**	**Regulation**	**Denatured Mol. mass**	**Tissue/ Cell**
I (nNOS)	soluble	Ca^{2+}-CaM-dependent	168 kDa	Brain; Skeletal muscle
II (iNOS)	soluble	Ca^{2+}-independent induced by LPS, cytokines.	130 kDa	Macrophages; VSM, cardiac muscle, many others ..
III (eNOS)	particulate + soluble	Ca^{2+}-CaM-dependent	135 kDa	Endothelial cells Cardiac muscle

CaM = calmodulin; VSM =vascular smooth muscle; LPS = lipopolysaccharide

reached a maximum in the left ventricle 6 hr after endotoxin injection (Schulz *et al.*, 1992).

b. Gene expression of NOS:

The endothelial NOS (eNOS) is a 135 kDa enzyme that is found primarily in the particulate fraction (Forstermann *et al.*, 1991). The gene for human eNOS contains 26 exons spanning approximately 21 kb of genomic DNA and encodes a mRNA of 4052 nucleotides located to the 7q35 →7q36 region of chromosome 7 (Sessa *et al.*, 1992; Marsden *et al.*, 1993). Release of NO through eNOS can be upregulated by various factors such as increases in shear stress (Buga *et al.*, 1991) exercise (Sessa *et al.*, 1994), pregnancy, and estradiol administration in male or female guinea-pigs (Weiner *et al.*, 1994). Exposure to inflammatory cytokines downregulates the expression of eNOS by destabilizing the mRNA for the enzyme and consequently, reducing its half-life (Yoshizumi *et al.*, 1993).

Isoform II, or iNOS, is a 130 kDa protein and has about 51% sequence identity with eNOS. The mRNA for iNOS from murine macrophages is 4-5 kb in size (Xie *et al.*, 1992). Calmodulin is tightly bound to iNOS as one of its constitutive units (Cho, *et al.*, 1992). The gene encoding human iNOS has been localized to the 17q11.2→q12 region of chromosome 17 (Marsden, *et al*, 1994). Agents which elevate cAMP levels (forskolin, isoproterenol) or mimic the action of cAMP (di-butyryl cAMP) synergistically enhance iNOS mRNA and protein levels in cytokine-treated endothelial and vascular smooth muscle cells (Durieu-Trautmann *et al.*, 1993).

c. Inhibitors of NOS activity and synthesis :

Enzyme activity in all isoforms of NOS can be inhibited by L-arginine analogues such as N^G-nitro-L-arginine methyl ester (L-NAME), N^G-monomethyl-

L-arginine (L-NMMA), and nitro-L-arginine (L-NA). These act as competitive inhibitors of all isoforms of NOS (Moncada *et al.*, 1991). There also are effective inhibitors of the expression, but not the activity, of iNOS. Example of these inhibitors include the glucocorticoids (e.g. dexamethasone) (Radomski *et al.*, 1990), the protein synthesis inhibitor, Cx, transforming growth factor-β, platelet-derived growth factor, interleukin-3, interleukin-4, and interleukin-10 (Sessa, 1994). Cx inhibits iNOS mRNA expression in a dose-dependent manner when added before exposure to pro-inflammatory cytokines or bacterial lipopolysaccharide. However, when Cx is added after immune stimulation, it enhances iNOS gene transcription by reducing the rate of decay of iNOS mRNA (Geller *et al.*, 1993; Evans *et al.*, 1994).

4. Potential roles of NO in the heart :

a. Role of NO in physiology of the heart :

In the cardiovascular system, NO is a major determinant of basal vascular tone, prevents platelet activation, limits leukocyte adhesion to vascular endothelium, and regulates myocardial contractility (Forstermann *et al.*, 1993; Loscalzo and Welch, 1995). There is a continuous regulation of coronary flow by a basal release of NO (pmoles) due to the activity of eNOS which is activated by increases in intracellular calcium within the physiological range (Moncada *et al.*, 1991). Heart cells synthesize NO at a substantial rate and, interestingly, degrade NO at a 100 fold higher rate than in isolated blood vessels (Kelm and Schrader, 1990). This may be a protective mechanism of the heart to limit the negative inotropic action of NO (Schulz and Triggle, 1994). Brady *et al.*, (1993) have shown *in vitro* that NO, and also sodium nitroprusside (a direct NO donor) cause a substantial reduction in cardiac myocyte contractile function via a cGMP-

dependent mechanism. Production of cGMP can occur through activation of the L-arginine-NO pathway with a subsequent direct activation of guanylate cyclase by NO (Moncada *et al.*, 1991). As a second messenger, cGMP interacts with at least three types of intracellular receptor proteins: cGMP-dependent protein kinase, cGMP-regulated ion-gated channels, and cGMP-regulated cyclic nucleotide phosphodiesterase (Lohmann *et al.*, 1991). Mery *et al* (1993) showed that 3-morpholino-sydnonimine-1, a NO donor, strongly reduced L-type calcium current which was previously elevated by cAMP, forskolin, or isoproterenol in frog ventricular myocytes (Mery *et al.*, 1993). There are also data showing that NO may affect diastolic heart function. The administration of sodium nitroprusside or the induction of iNOS with interleukin-1 have been shown to abbreviate contraction and hasten relaxation in ferret papillary muscles (Evans *et al.*, 1993), in the isolated ejecting guinea-pig heart (Grocott-Mason *et al.*, 1994), and in humans (Paulus *et al.*, 1994). In these latter two studies, there was only a small increase in peak systolic pressure and no effect on left ventricular dP/dt caused by addition of exogenous NO.

b. Role of NO in pathophysiology of the heart :

Although NO is essential for normal physiological function in providing a patent coronary circulation, the release of excess quantities of NO following the expression of iNOS is detrimental to heart function (Schulz *et al.*, 1992; Schulz *et al.*, 1995). Elevations of cGMP levels were observed in cardiac myocyte preparations incubated with cytokines (Kinugawa *et al.*, 1994) or in homogenates of left ventricle isolated from endotoxaemic rats (Schulz *et al.*, 1992). Exposure of cardiac myocytes to LPS and/or pro-inflammatory cytokines, caused an enhanced iNOS activity (Schulz *et al.*, 1992) and depression in their contractile function (Ungureanu-Longrois *et al.*, 1995b). Recently, Schulz *et al.*

(1995) showed that perfusion of isolated working rat hearts with interleukin-1β and tumor necrosis factor-α caused a delayed depression of myocardial contractile function. This loss of function was associated with an expression of iNOS activity which was prevented by the protein synthesis inhibitor, Cx (Schulz *et al.*, 1995).

Overproduction of NO may be involved in the pathological alterations leading to the development of heart failure and systemic inflammatory response syndrome (Ungureanu-Longrois *et al*, 1995a). These may include the development of cardiac depression associated with anti-cancer cytokine therapy (Deyton *et al*., 1989), septic shock conditions in immune activated states (Suffredini *et al.*, 1989), myocarditis, endocarditis, allograft rejection (Tanaka *et al.*, 1995), and idiopathic dilated cardiomyopathy (de Belder *et al.*, 1993).

c. Molecular targets of nitric oxide :

Nitric oxide, once produced by the cell that generates it has a number of specific targets both intra- and extracellularly. These include proteins with a heme group such as guanylate cyclase, hemoglobin, and NADPH-ubiquinone, or proteins with an iron-sulfur centre such as *cis*-aconitase, and ribonucleotide reductase (Moncada *et al.*, 1991; Lowenstein *et al.*, 1994). NO can affect cells by facilitating the transfer of an ADP-ribose group to an accepting molecule through the activation of ADP-ribosyltransferase enzyme within the cell (Brune and Lapetina, 1989). NO can combine readily with superoxide anion to form peroxynitrite ($ONOO^-$) (Beckmann *et al.*, 1990) which can act as an NO-donor in the biological system to cause vascular smooth muscle relaxation (Moro *et al*., 1995). Peroxynitrite is protonated at physiological pH and then rapidly decomposes to yield strong oxidants and highly reactive species (Beckmann *et*

al., 1990) Peroxynitrite has been implicated in membrane lipid peroxidation and protein oxidation reactions in biological systems (Radi *et al.*, 1991).

5. Mechanics of heart failure :

Heart failure is a syndrome that results from left ventricular dysfunction after mechanical overload, myocardial damage with cell loss, or a combination of the two. These processes initially lead to compensatory hypertrophy, adaptive remodelling of the ventricle, and concomitant changes in systolic and diastolic function. When systolic dysfunction occurs, the ability of the myofibrils to shorten against a load or generate pressure, is defective (McCall *et al.*, 1993). There is evidence that the failing human left ventricle *in vivo* subjected to additional preload is unable to use the Frank-Starling mechanism because of impairment of the force-tension relation (Schwinger *et al.*, 1994). It was suggested that this phenomenon could be explained by a failure of the myofibrils to increase the Ca^{2+} sensitivity after an increase of the sarcomere (contractile unit) length (Schwinger *et al.*, 1994).

6. Beta-adrenergic system and heart function :

There are two types of β-adrenergic receptors that are known to exist in the heart, β_1 and β_2 receptors. Both types of receptors are found in the ventricles and atria. Activation of these receptors leads to an increase in cAMP levels, and rise in intracellular Ca^{2+} concentration and consequently, increase the inotropic state of the myocardium. There are different factors that play a role in altering the β-adrenergic-cAMP pathway. In mammalian hearts, increase in cGMP levels activates a cGMP-specific protein kinase which in turn reduces cAMP-elevated calcium influx through phosphorylation of the L-type Ca^{2+}-channel (Mery *et al.*,

1991). Thus, it was suggested that cytokine exposure of cardiac muscle that occurs during septic shock may cause, not only cardiac depression, but also alterations in the β-adrenergic response of cardiac myocytes (Lange and Schreiner, 1992). Gulik *et al.* (1991) demonstrated that neonatal rat cardiac myocytes, exposed to medium from activated splenocytes, lost their contractile response to β-adrenergic stimulation. The depressent effect of cytokines seems to be related to an uncoupling of β-adrenergic receptors from adenylate cyclase, and is not due to changes in receptor number, affinity or function, nor due to changes in adenylate cyclase activity, since the response to forskolin, a direct activator of adenylate cyclase (Metzger and Lindner, 1981) was maintained (Lange and Schreiner, 1992). Lange and Schreiner (1992) suggested that this decreased responsiveness to β-adrenergic agonists is possibly due to a decrease of cAMP levels. However, the mechanisms by which cytokines affect cAMP generation and/or degradation in cardiac myocytes are still unclear. In isolated ventricular myocytes from normal adult rats, Balligand *et al.* (1993) showed that inhibition of NOS by L-NMMA had little or no effect on basal contractile state, as assessed by the amplitude of myocyte motion, but significantly enhanced the positive inotropic response to the β-adrenergic agonist isoproterenol. A selective depression in β-adrenergic responsiveness occurs in cardiac myocytes following interleukin-1β, tumor necrosis factor-α and interferon–γ treatment which is reversible with the NOS inhibitor L-NMMA (Ungureanu-Longrois *et al.*, 1995b). Recently Pyo and Wahler (1995), studied the dose-response to isoproterenol in ventricular myocytes isolated from rejecting rat cardiac allografts. In this study, they showed that these cells exhibited a large reduction in the β-adrenergic contractile response to isoproterenol. In perfused heart preparations isolated from septic rats, basal rates for isoproterenol response were elevated and the EC_{50} for

the chronotropic response to isoproterenol was significantly less than in atria from control animals indicating that chronotropic sensitivity to β-adrenergic stimulation is increased in sepsis (Smith *et al.*, 1986).

The basal release of physiological quantities of NO via stimulation of the Ca^{2+}-dependent NOS plays a major role in regulating vascular tone and maintaining a patent coronary circulation. However, excess release of NO through the induction of iNOS in isolated working hearts is detrimental to cell function and may lead to a series of pathological disorders including myocardial depression and alterations in the β-adrenergic/cAMP pathway through mechanisms which are a focus of this thesis.

B. THESIS HYPOTHESIS

Myocardial depression in isolated working rat hearts is associated with the induction of the Ca^{2+}-independent NO synthase (iNOS) in a time-dependent manner.

C. THESIS OBJECTIVES

The purpose of this study is to investigate the factors influencing myocardial depression in isolated working rat hearts over time. In this study, the role of perfusate calcium concentration, energy substrate, and involvement of iNOS expression in cardiac depression were investigated. The following objectives were studied using the isolated working rat heart:

1. To compare the effects of pyruvate and the free fatty acid palmitate as energy substrates in the heart and their effects on cardiac mechanical function over 2 hr of *in vitro* perfusion.

2. To analyze the time-dependent effects of perfusate free calcium concentration on cardiac mechanical performance.

3. To determine the actions of the protein synthesis inhibitor, Cx, on cardiac mechanical performance over time.

4. To determine whether there is an enhancement of iNOS activity and expression of iNOS protein in hearts under these perfusion conditions.

5. To determine the time-dependent changes in dose-response to isoproterenol on cardiac work hearts perfused with different perfusate conditions.

CHAPTER II

MATERIALS AND METHODS

MATERIALS

Compound	Source
Acrylamide	GIBCO BRL
bis-acrylamide	GIBCO BRL
AG 50W-X8 resin (200-400 mesh)	Bio-Rad
Ammonium persulfate	GIBCO BRL
Aprotinin	Sigma
Bichinchonic acid	Sigma
Bovine serum albumin (Fraction V)	Boehringer Mannheim
Bromophenol blue	BDH Biochemicals
L-citrulline	Sigma
Copper (II) sulphate	Sigma
DL-dithiothreitol	Sigma
ECL reagent	Amersham
Na_2EDTA	BDH Biochemicals
EGTA	BDH Biochemicals
E-toxate kit	Sigma
Glycerol	BDH Biochemicals
HEPES	BDH Biochemicals
Insulin-Toronto (beef and pork)	Novo Lab. Limited
Isoproterenol	Sigma
Leupeptin hemisulfate salt	Sigma
β-mercaptoethanol	BDH Biochemicals
L-NMMA	Alexis Corp./Wellcome Research Laboratories

Compound	Source
L-[U-^{14}C]-arginine	Amersham
L-valine	Sigma
NADPH	Boehringer Mannheim
Palmitate, sodium salt	Sigma
Phenylmethylsulfonyl fluoride	Sigma
Pyruvate, sodium salt	Sigma
scintillation liquid (Scintisafe)	Fischer Scientific
SDS	BDH Biochemicals
sodium pentobarbital (Euthanyl™)	Abbott
TEMED	GIBCO BRL
Tris-base	GIBCO BRL
Tris-HCl	Boehringer Mannheim
trypsin inhibitor	Sigma

METHODS :

Heart Perfusions :

a. Animal Preparation : The procedures for animal handling and heart isolation were approved by the University of Alberta Health Sciences Animal Welfare Committee.

Male Sprague-Dawley rats weighing 250-300g were anesthetized i.p. with 50mg/kg body weight of sodium pentobarbital (Euthanyl™). A transverse incision was made to open the abdominal cavity. The diaphragm was transected and the chest was opened by an incision cut along the sternum. The pericardium was cut and pulled away from the heart. The heart was picked up and pulled back and then excised with the lungs and quickly rinsed in ice-cold Krebs' solution for few seconds. The heart was picked up by the aorta which was opened wide and inserted onto the aortic cannula and tied firmly. A sketch of a cannulated working heart is shown in Figure 2.1.

b. Langendorff Perfusion : Retrograde perfusion as described by Langendorff was initiated immediately after cannulation with modified Krebs'-Henseleit solution (pH 7.4) at 37°C gassed with 95% O_2-5% CO_2 containing 11mM glucose, 0.5 mM EDTA and either 1.75 mM or 3.0 mM total calcium chloride. This solution was used as a short-time perfusion buffer while the heart was prepared as a working heart preparation. The pulmonary artery was carefully snipped to allow free outflow of the coronary circulation. The perfusion was continued for approximately 10 minutes from a reservoir placed to give a hydrostatic pressure equivalent to 60 mmHg. The Langendorff perfusion allows for the wash-out of blood and traces of anesthetic as well as equilibration of the buffer substrate concentrations with those in the interstitial fluid. This retrograde perfusion is

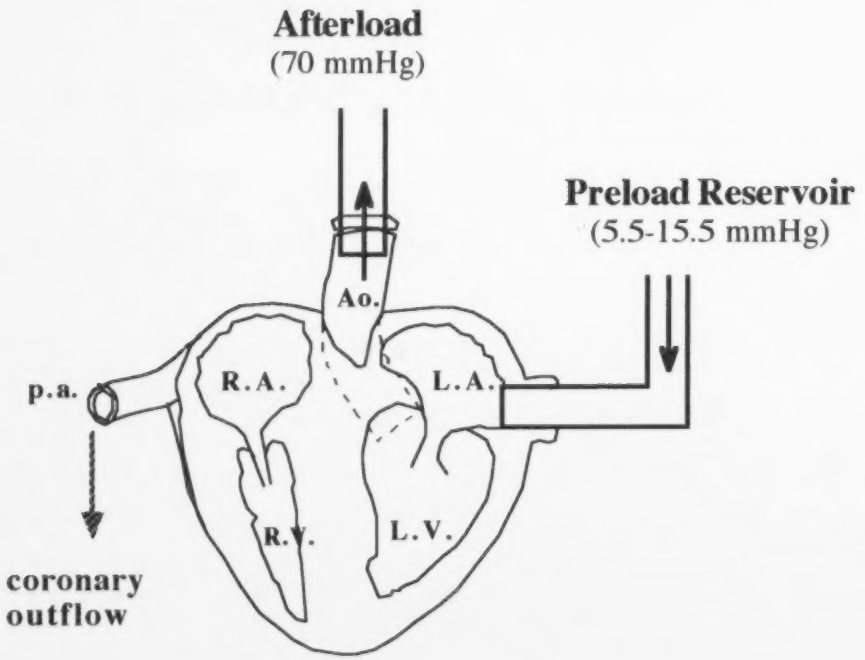


Figure 2.1 A schematic drawing of an isolated working heart. The heart is cannulated via the left atrium (L.A.) that receives perfusate from a preload reservoir which can be set to give different preload pressures. The left ventricle (L.V.) then ejects the perfusate via the aorta (Ao) which is connected to a height of tubing that forms the afterload as mentioned in Methods. Coronary outflow is allowed via the pulmonary artery (p.a.) and pours into a collecting reservoir for recirculation. The arrows indicate the direction of the flow of perfusate within the heart during the working mode. R.A., right atrium; R.V., right ventricle.

important to allow the heart to recover from the brief period of hypoxia that was associated with excision and initiation of perfusion.

c. Working Mode Perfusion : The isolated working heart method was originally described by Neely *et al.* (1967). During this time, the heart is rotated on the aortic cannula in order to position the atrial cannula close to the opening of the pulmonary vein into the left atrium. Any pieces of lungs, connective tissue, or thymus were cut and removed to clear out and expose the left atrial opening for cannulation. Once the left atrium was cannulated with the heart hanging free and beating regularly (10 minutes), the retrograde Langendorff perfusion was stopped and, simultaneously, the working perfusate was allowed to enter into the left atrium under a hydrostatic filling pressure, or preload, of 9.5 mmHg from a preload oxygenator-reservoir set at a level from the heart and can be placed at different levels to give preload pressures ranging between 5.5 to 15.5 mmHg. The coronary effluent emptied into a reservoir which was originally filled with 100 mL of working buffer consisting of Krebs'-Henseleit solution containing 11mM glucose, 100μU/mL insulin, 0.5mM EDTA and with one of the following additions:

- 1.75 mM total calcium chloride (1.25mM free Ca^{2+}), 5 mM pyruvate (as energy substrate) and 0.2% bovine serum albumin (BSA).
- 1.75 mM total calcium chloride (1.25mM free Ca^{2+}), 0.8 mM palmitate (as energy substrate) prebound with 3% BSA
- 3.0 mM total calcium chloride (2.5mM free Ca^{2+}), 0.8 mM palmitate (as energy substrate) prebound with 3% BSA

The working buffer was recirculated by a peristaltic pump (Model 7553-70, Cole-Parmer Instrument Corporation systems) into the preload reservoir before its entry to the heart. Buffer entering the left atrium from the preload oxygenator-

reservoir passed into the left ventricle and ventricular contraction forced the perfusate into a pressure chamber attached to the aortic cannula. About 1-2 mL of air was trapped within the chamber to provide some elasticity for aortic pressure. The perfusate ejected through the aortic root was pumped by the left ventricle through a length of tubing to a height corresponding to a hydrostatic pressure afterload of 70 mmHg (comparable to the peripheral resistance in the rat under *in vivo* conditions). Under these conditions the isolated heart was functioning in the working mode (Figure 2.1). To measure the parameters of the working heart, an ultrasound flow probe (2N Series, Transonic Systems Inc.) was connected to the preload line to measure cardiac output (venous return) and another flow probe was connected to the afterload line to measure the aortic flow. The flow rates from the flow probes were displayed in mL/min by an electronic flowmeter (Model T206, Transonic Systems Inc.). The aortic line was also connected to a pressure transducer (Model 1050 BP, BioPac Systems Inc.) that measures the change in ventricular pressure (peak systolic and diastolic pressures) during each contraction cycle which were amplified and displayed using a Grass Model 7D physiograph. All the buffer reservoirs were water-jacketed to keep the perfusate temperature constant at 37°C throughout the perfusion protocol. Following 5 minutes in the working mode, hearts were electrically paced at 300 beats/min using a Grass Model SD9 stimulator (regular square wave stimuli, 0.6ms duration, 5 Hz) for the remainder of the experiment. Equilibrium was reached in all parameters of the isolated working heart within 10 minutes prior to the start of the functional study protocols.

2. Functional Study Protocols :

a. Preload Function studies: Once equilibrium in the working heart parameters (such as CO, peak systolic pressure, heart rate, coronary flow) was achieved, a preload function test was performed against a constant afterload of 70 mmHg. This was done by altering the preload pressure in increments of 2 mmHg starting from 5.5 to 15.5 mmHg. Peak systolic pressure, aortic flow and cardiac output was recorded at each preload pressure level. Upon completion of this test, the preload pressure was readjusted to 9.5 mmHg. From this point in time (defined as t=0 hr) the hearts were perfused for a 2hr period. At t=2hr, the preload function test was repeated to determine alterations in cardiac mechanical function over time. These preload function test gives an important estimation of the efficiency in the contractile machinery in the heart muscle functioning under these in vitro conditions. It tests the Frank-Starling mechanism by which cardiac muscle responds to changes in ventricular wall stress.

b. Time-Function study: A time-function study over a 2hr perfusion was performed from t=0hr under preload of 9.5 mmHg and afterload of 70 mmHg. Measurements of cardiac parameters were recorded at 10 minute intervals.

c. Isoproterenol Dose-Response Studies: Isoproterenol dose-response studies were carried out after the preload function tests either at t=0hr or at t=2hr in separate groups of hearts. Isoproterenol (from 10^{-12} to 10^{-7}M) was added to the recirculating buffer in cumulative doses. Measurements of cardiac parameters were recorded upon reaching stability following the addition of each dose of isoproterenol to the buffer. Cardiac work was used as an index of functional

response to cumulative doses of isoproterenol under constant workload conditions (*Preload*=9.5mmHg and *Afterload*=70mmHg). At the end of the isoproterenol dose-response studies, either after t=0hr or t=2hr, the ventricles were frozen using Wollenberger clamps cooled to liquid nitrogen temperature and stored at -80°C for further analytical and enzymatic studies. A summary of the perfusion protocol is given in Figure 2.2. Determination of the effective-concentration corresponding to 50% of maximal response (EC_{50}) is performed by computer-assisted estimation using GraphPad Inplot sofware facility gratefully provided by Dr. Clanachan in the Pharmacology department. A constant range (10^{-10} to 10^{-8}M) of the dose-response curve was chosen for all groups consistently.

3. Tissue Analysis :

a. Preparation of cytosolic fraction: The frozen ventricular tissues were pulverized in a pestle and mortar cooled to the temperature of liquid nitrogen. A portion of this tissue powder (150-200 mg) was placed in four volumes of ice-cold homogenization buffer (see below), containing phenylmethylsulfonyl-fluoride (protease inhibitor) and homogenized with an Ultra-Turrex (Model PRO 200) disperser using three strokes of 3s duration each. The homogenate was centrifuged at 100,000 ×g for 35 minutes at 4°C and the cytosolic fraction (supernatant) was kept on ice for immediate assay of NOS activity by the formation of radiolabelled L-citrulline from L-[U-^{14}C]-arginine (citrulline assay).

b. *Homogenization Buffer Preparation*: Prepare 10 mM HEPES, 0.32 M sucrose, 0.1 mM Na_2EDTA , and adjust to pH 7.2 with 2N NaOH, then add 1mM DL-dithiothreitol, 10μg/mL trypsin inhibitor, 10μg/mL leupeptin hemisulfate salt, and 2 μg/mL aprotinin. The buffer can be stored at -20°C after preparation.

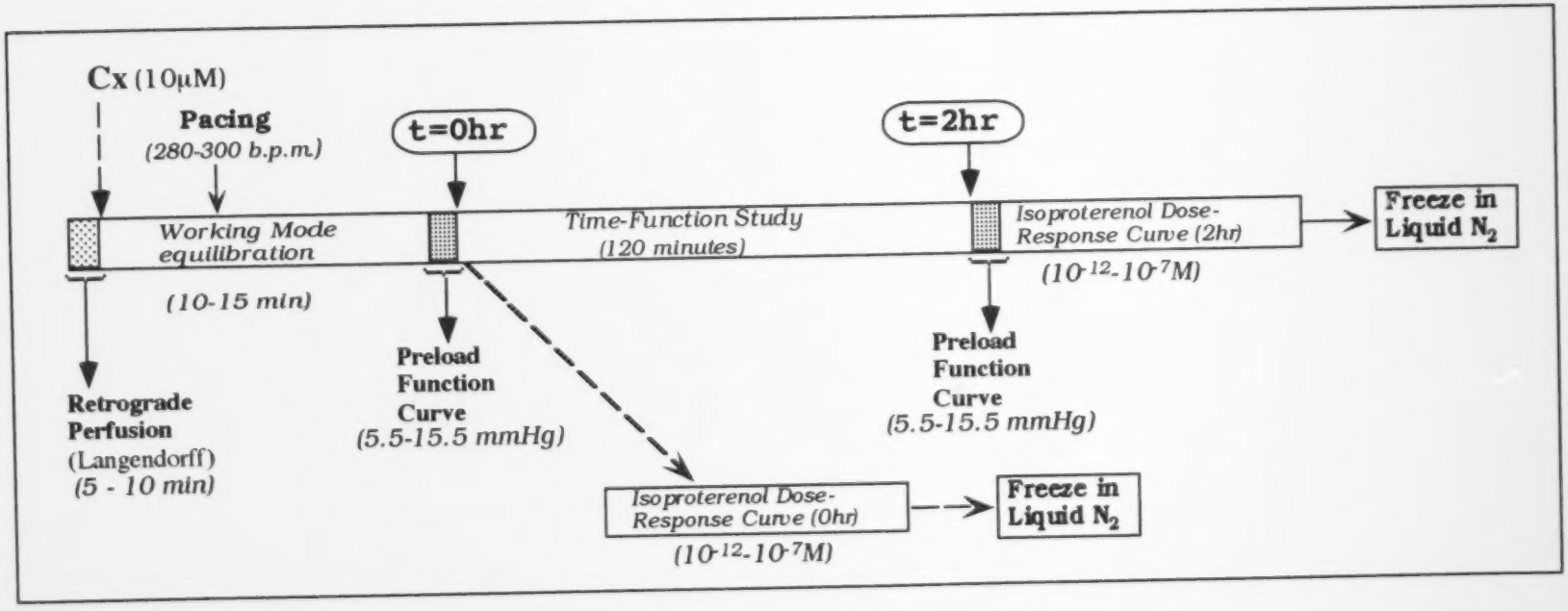


Figure 2.2 A summarized sketch describing the isolated working heart perfusion protocol. Retrograte perfusion (▨) was initiated immediately after heart isolation for 5-10 minutes and was followed by working mode perfusion that consisted of two functional studies : preload function curves (▤ , at t=0 hr and t=2 hr) and time-function study (□) for 2 hr prior to isoproterenol dose-response tests and freezing in liquid N_2 . All studies were performed at constant afterload of 70 mmHg. Cx was added to perfusate prior to working mode (see Methods for further details).

c. Citrulline Assay : This assay was used to determine both Ca^{2+}-dependent and Ca^{2+}-independent NOS activities in cytosolic fractions obtained from the ventricular wall. NO formation in the cytosolic fraction was measured by the formation of L-^{14}C-citrulline from L-[U-^{14}C]-arginine in the presence of saturating concentrations of the enzyme's cofactors as described previously by Schulz *et al.* (1995). Samples of cytosols were incubated as duplicates in cocktail assay buffer (see below) for 60 min at 37°C in the absence or presence of either 1mM EGTA, or EGTA plus 1mM L-NMMA (see section c-1 below).

c-1. Assay protocol : consisted of four major steps.

1. prepare tissue cytosols
2. prepare cocktail buffer
3. prepare resin
4. assay procedures

Step 1 : cytosolic fractions are prepared as mentioned above in section "a".

Step 2 : weigh out 70 mg of L-valine and dissolve in 10 mL of 50mM (pH 7.2) KH_2PO_4 buffer containing 1.2mM $MgCl_2$, 0.24mM $CaCl_2$. Add NADPH (125 μM), L-citrulline (1.25 mM), L-arginine (22.5 μM), BH_4 (12.5 μM), DL-dithiothreitol (1.25 mM) and 50 μCi/mL (2.5 μM) L-[U-^{14}C]-arginine-HCl. Mix well and keep on ice.

Step 3 : Weigh out approximately 1g of AG 50W-X8 resin for every 3 samples used in the assay. Add 60mL of 2N NaOH to the resin in a glass beaker to activate the resin. Swirl and allow to settle then decant the NaOH solution and wash at least three times with deionized distilled water (DDW) until pH measures ≤7.0. After decanting the last wash, add a measured amount of DDW

(1mL/sample) mix and again measure pH, to ensure neutral or slightly acidic pH.

Step 4: Number 2mL microcentrifuge tubes and assemble into rack. Samples are prepared in duplicates and each tissue sample requires a set of 3 duplicates (6 tubes):

- pipet 100μL of cocktail buffer directly into the bottom of each tube
- pipet 5μL of EGTA into tubes 3 and 4 of each set of six tubes
- pipet 5μL of EGTA + L-NMMA into tubes 5 and 6 of each set.
- place tubes in water bath at 37°C. Pre-incubate for 10 minutes.
- add 20μL of cytosol into each of six tubes, vortex briefly and then incubate for 60 minutes at 37°C.
- After incubation, add 1mL of activated resin slurry to tubes to stop the reaction (at neutral pH, resin quantitatively absorbs L-arginine which exists in cationic form whereas L-citrulline remains in solution). Remove tubes and place at room temperature.
- Add 300μL of DDW to each tube. Cap and vortex briefly.
- spin tubes at 14,000 r.p.m. for 2 minutes at room temperature.
- carefully remove 700μL of supernatant from each tube and place into small counting vial.
- add 2mL of scintillation liquid (Scintisafe, Fischer) to each vial and count as ^{14}C for 5 minutes.

First duplicate (tubes 1&2) is assigned to determine the total NOS activity, the second duplicate (tubes 3&4) is for Ca^{2+}-dependent NOS activity after subtracting the average counts from the first duplicate and the third duplicate (tubes 5&6) for Ca^{2+}-independent activity after subtracting the average counts from the second duplicate.

d. Protein Determination: Protein content of the cytosolic fraction was determined by the method of Bradford (1976) using BSA as a standard. A volume (10μL) of cytosol diluted with DDW (1:20) was mixed with 200μL of bichinchonic acid reagent. The bichinchonic acid reagent was prepared freshly by mixing bichinchonic acid solution with copper (II) sulphate in a 50:1 ratio. Dilutions of BSA in DDW were prepared along with the cytosols in order to determine a standard curve. All samples were pipetted into the wells of a microplate and incubated at 37°C for 30 minutes. Immediately following incubation the optical density was read for all samples at 560nm using an UV-Max microplate reader. Protein contents were calculated from the BSA standard curve and expressed in mg protein.

4. Determination of perfusate endotoxin level : Samples of perfusate were measured for endotoxin content with an E-toxate kit (Sigma) which is based on the *Limulus* amoebocyte lysate assay. The origin of this assay came from the work of Levin and Bang (1968). E-toxate is prepared from a lysate of the circulating amebocytes of the horseshoe crab, *Limulus polyphemus*. When exposed to minute quantities of endotoxin (LPS), the lysate increases in opacity as well as viscosity and may gel depending on the concentration of endotoxin. The mechanism for this reaction involves two steps :

1. Activation of trypsin-like, preclotting enzyme(s) by endotoxin in the presence of Ca^{2+} ions.
2. The activated enzyme(s) then modify a "coagulogen" by limited proteolysis to produce a clottable protein.

Endotoxin standard dilutions were prepared in endotoxin-free DDW in sterile, capped polystyrene tubes and used as a reference to determine endotoxin

contents in unknown samples. A negative control was prepared by using endotoxin-free water alone. samples were incubated for 1hr and tubes were gently inverted 180 degrees while observing for evidence of gelation. A positive test is the formation of a hard gel which permits complete inversion of the tube or vial without disruption of the gel. All other results: soft gels, turbidity, increase in viscosity, and clear liquid are considered negative. Semi-quantitative analysis to determine LPS content was performed on samples that yielded positive results by making 1:10 dilutions of these samples in endotoxin-free water and testing each dilution until a negative test result is obtained. The endotoxin level expressed in endotoxin units per mL (EU/mL) is then derived by multiplying the inverse of the highest dilution of sample found positive (HD+) by the lowest concentration of endotoxin Standard found positive (LC+):

Endotoxin (EU/mL) = (HD+) × (LC+)

Interpretation of results: (+) = hard gel

(-) = Absence of hard gel

5. Western Blotting:

a) SDS-PAGE:

Freshly prepared ventricular cytosols were treated with 1:1 v/v of a buffer containing 0.062M Tris-HCl (pH 6.8), 10% glycerol, 2% SDS (w/v), 1% β-mercaptoethanol, and 3.3mg/100mL bromophenol blue. The mixture was boiled for 5 minutes only to denature the proteins and stored at -20°C for SDS-PAGE the next day. Equal amounts (90μg) of the denatured proteins were loaded per lane and separated on a 9% SDS-polyacrylamide gel using a Mini Protean II apparatus (Bio-Rad). Samples (5-10μL) of high range prestained molecular

weight standard markers (48-199 kDa, Bio-Rad) were loaded and run with the sample proteins for separation. When separation of samples within the gel was complete, the gel was removed and sandwiched to an equal sized nitrocellulose membrane (0.45 micron, Bio-Rad) and assembled into the transfer apparatus (Bio-Rad) where the samples were allowed to transfer for 2hr at 4°C under constant voltage (100V). After transfer is complete, the membrane was removed and kept between two pieces of filter paper (3 mm Whatmann) allowing it to dry overnight prior to immunoblotting the next day. The gel was stained for 30 min with Coomasie blue stain (1gr R250 Coomassie Blue, 500mL 2-propanol, 200mL acetic acid, 1300mL DDW in 1.5L total volume) and then destained with the Coomassie Destain solution (7.5% acetic acid, 10% methanol) for 3 hr to visualize the remaining untransferred proteins. This procedure was helpful to determine the efficiency of the transfer protocol.

b) Immunoblotting:

On the day following SDS-PAGE, the membrane was blocked for 3-5 hr with 10% milk in PBS solution at room temperature. The membrane was then incubated for 3 hr at room temperature with immune serum (1:400 dilution in 1% milk PBS solution) containing rabbit polyclonal primary antibodies raised against rat and mouse iNOS (Merck Research Laboratories). After three washes of 5 minutes each, twice with PBS/ 0.05% Tween 20 and once with PBS alone, the membrane was incubated for 1hr at room temperature with goat anti-rabbit IgG-Horse Raddish Peroxidase conjugate (Santa Cruz Labs) at a 1:2000 dilution in 1% milk PBS solution. The membrane was washed 3-5 times with PBS/0.05% Tween 20 and 2 times with PBS alone. Finally, equal volumes of ECL reagent (Amersham) was added the surface of the membrane for one minute to activate

fluorescence on the horse raddish peroxidase conjugate to the secondary antibodies prior to X-ray exposure. After activation of fluorescence, the membrane was covered with Saran Wrap and taped on the X-ray cassette screen and exposed for 30 seconds to an X-ray film (Kodak, 8.5×11 in.) within 15 minutes of fluorescence activation with the ECL reagent.

Statistical Analysis: Results were expressed as mean ± SEM for n experiments. As appropriate, Student's paired or unpaired *t* test or one-way ANOVA were used for statistical comparisons. $P<0.05$ was used as the criterion for statistical significance.

CHAPTER III

RESULTS

1. *Effects of pyruvate and palmitate on cardiac performance over time*:

Effects of the presence of different energy substrates have been investigated in isolated working rat hearts over time. Figure 3.1 shows the effects of pyruvate and palmitate on cardiac mechanical function over 2hr of perfusion under identical calcium concentration (1.25 mM free Ca^{2+}). All comparisons were tested using Students *t* test. Hearts perfused with pyruvate (n=5) showed a 24% loss in function as cardiac work after 2h of perfusion as compared to starting conditions at t=0 hr. However, the palmitate-perfused group (n=9) showed a significant loss of 49% ($p<0.001$) of mechanical function as compared to starting conditions at t=0 hr. Cardiac function was stable for about 80 minutes in both groups and was not significantly different between the two groups (Table 3.1). Both groups showed a difference in cardiac work at the beginning of the perfusion protocol but not statistically significant (Table 3.1). Measuring coronary flow over time in the two groups showed that coronary flow varied within a narrow range (20-25 mL/min) and was not significantly different at 2hr except for the low calcium palmitate perfused group (Table 3.2: **c** *vs.* **d**, n=9, $p<0.01$). Thus the replacement of pyruvate with palmitate did not prevent the loss of function in isolated working rat heart perfused for 2hr.

2. *Effects of pyruvate and palmitate on cardiac response to preload changes over time*:

Figure 3.2 shows the response of hearts perfused with either pyruvate or palmitate in buffer containing 1.25mM Ca^{2+} to changes in preload pressure

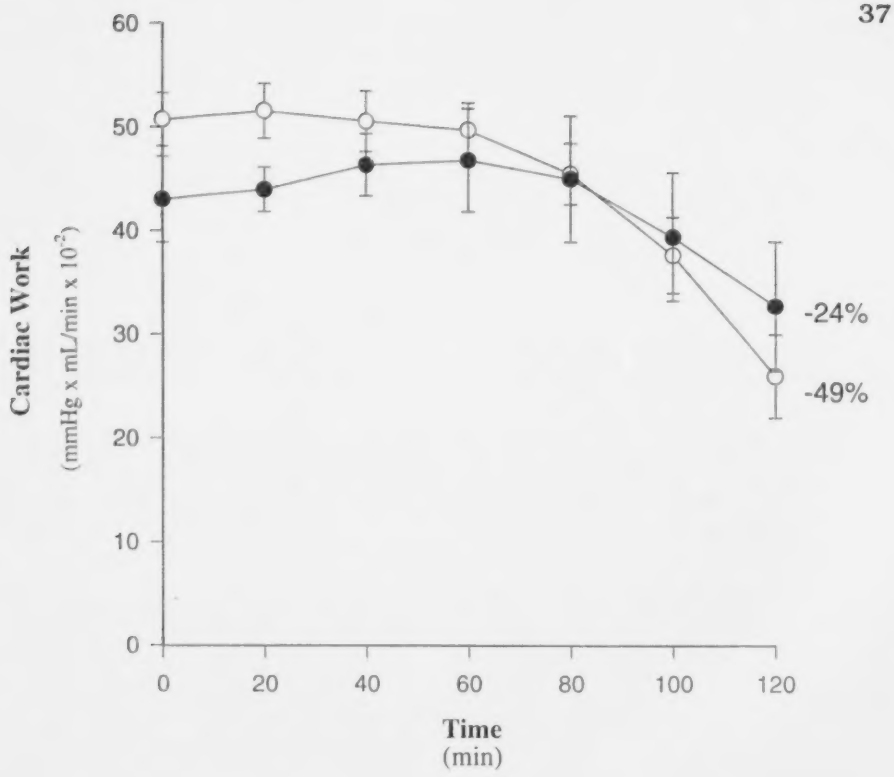


Figure 3.1 Effects of pyruvate and palmitate on cardiac work over 2hr of perfusion. Variation in function is given as cardiac work with respect to time of perfusion protocol. (●) represents hearts (n=5) perfused with 1.25 mM Ca^{2+} Krebs-Henseleit buffer containing 5mM pyruvate as the energy fuel source. This group showed a 24% loss of function after 2hr of perfusion compared to starting conditions at t=0 hr, although the difference was not statistically significant between 0 and 2hr. The second graph (○) represents hearts (n=9) perfused with 1.25mM free Ca^{2+} buffer containing 0.8mM palmitate. This group showed a significant loss of function as cardiac work (-49%) over 2hr compared to starting conditions at t=2hr. Therefore replacing pyruvate with palmitate did not reduce the loss of function over time in the isolated working heart.

Table 3.1 Cardiac Work values at 0, 1 and 2hr of perfusion under various buffer conditions. All groups showed similar functions during the first hour of perfusion (1 hr) compared to the function at the beginning of perfusion (0 hr) which also were not significantly different among the four groups studied (**a**, **c**, **e**, and **g**). At the end of perfusion (2 hr), there was a significant loss in function as cardiac work in both groups perfused with either low or high Ca^{2+} in palmitate buffer compared to their corresponding starting conditions (**c** *vs.* **d** and **e** *vs.* **f**; $p<0.05$). Addition of Cx to low Ca^{2+}/palmitate buffer prevented the loss of function over 2 hr of perfusion (**d** vs. **h**; $p<0.01$). Cardiac works in all groups were studied under constant workload conditions (Preload=9.5 mmHg; Afterload=70 mmHg). Comparisons among other combinations showed no significant differences using Student's *t* test.

CARDIAC WORK

(mmHg . mL/min .10^{-2})

	0 hr	**1 hr**	**2 hr**
1.25mM free Ca^{2+}/**0.8**mM pyruvate (*n=5*)	43.0 ± 4.1[a]	46.8 ± 5.0[i]	32.7 ± 6.2[b]
1.25mM free Ca^{2+}/**0.8**mM palmitate (*n=9*)	50.7 ± 2.6[c]	49.7 ± 2.6[j]	26.0 ± 4.0[d]
2.5mM free Ca^{2+}/**0.8**mM palmitate (*n=9*)	53.9 ± 2.3[e]	53.7 ± 2.6[k]	37.2 ± 5.3[f]
1.25mM free Ca^{2+}/**0.8**mM palmitate, **10**μM Cx (*n=6*)	48.8 ± 4.2[g]	50.0 ± 4.2[l]	45.5 ± 5.0[h]

Table 3.2 Coronary Flow at 0 and 2hr in groups perfused with different buffer conditions. In all groups studied, coronary flow varied within a narrow range (20-25 mL/min) and was not significantly different among various groups. However, these groups did not show a significant change in coronary flow after 2hr of perfusion except for the low calcium, palmitate group which was significantly different at 2hr compared to the starting conditions at 0hr within the same group (**c** *vs.* **d**; $p<0.01$, n=9). Student's *t* test was used for statistical comparisons among the groups.

	Coronary Flow (mL/min)	
	0 hr	**2 hr**
1.25mM free Ca^{2+}/ **5**mM pyruvate (*n=5*)	22.8 ± 1.4[a]	19.8 ± 2.3[b]
1.25mM free Ca^{2+}/ **0.8**mM palmitate (*n=9*)	24.1 ± 0.8[c]	20.1 ± 1.1[d]
2.5mM free Ca^{2+}/**0.8**mM palmitate (*n=9*)	26.7 ± 1.2[e]	22.8 ± 1.7[f]
1.25mM free Ca^{2+}/**0.8**mM palmitate,**10**μM Cx (*n=6*)	22.8 ± 1.1[g]	22.7 ± 2.8[h]

(5.5-15.5 mmHg) measured at t=0 and 2hr of perfusion. At t=0 hr hearts perfused with either pyruvate or palmitate showed an increase in mechanical function with each increase in preload pressure. However, after 2hr of perfusion this response was markedly reduced in palmitate perfused hearts at all preload pressures ($p<0.001$), whereas the loss in mechanical response was not significant in the pyruvate perfused hearts.

3. *Effects of buffer calcium levels on cardiac performance over time*:

Figure 3.3 shows the response given as cardiac work of two groups of hearts perfused with palmitate under either low calcium (1.25 mM free Ca^{2+}) or high calcium (2.5mM free Ca^{2+}) conditions over 2hr perfusion. At 0hr, cardiac performance was similar in the low and high calcium perfused groups of hearts (**c** *vs.* **e**, Table 3.1). This function was stable for at least within the first hour (1 hr) of perfusion in both groups. Both groups, high and low calcium, showed significant loss in function (31%, n=9; and 49%, n=9 respectively) at t=2hr compared to starting conditions within each group. The high calcium group showed an improvement of cardiac mechanical function over time compared to the lower calcium group with no statistically significant difference (Student's *t* test) between the two groups at t=2 hr. There was no significant change in coronary flow between 0 and 2hr in all groups except for the 1.25mM Ca^{2+}/palmitate group, where there was a significant decline in coronary flow by the end of the experiment (Table 3.3). The improvement of function seen in the high calcium palmitate perfused group was independent of changes in coronary flow.

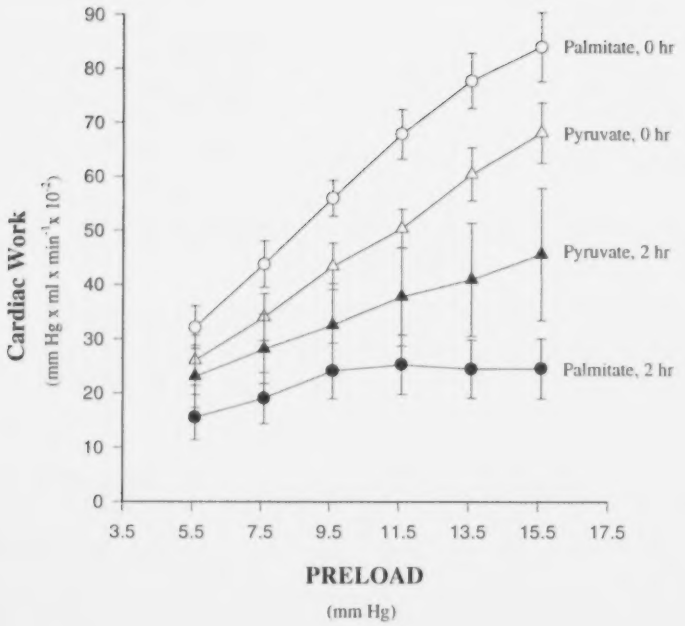


Figure 3.2 Relationship between cardiac work and preload pressure in hearts perfused with either pyruvate or palmitate buffers containing 1.25mM Ca^{2+} at 0hr and after 2hr perfusion. At t=0 hr, both groups perfused with either pyruvate (△) or palmitate (○) showed an increase in response given as cardiac work with every increase in preload pressure from 5.5 to 15.5 mmHg. After 2hr of perfusion, the response to preload changes was not significantly altered in the pyruvate (▲) group (n=5) compared to t=0 hr within the same group. However, the palmitate perfused group (●) showed a significant loss of response to preload changes after 2 hr of perfusion ($p<0.01$, n=9). The buffer in both groups contained equal concentration of calcium (1.25mM free Ca^{2+}). Therefore, the presence of palmitate as energy fuel source in the perfusate did not have an advantage over pyruvate in the isolated working heart perfusion.

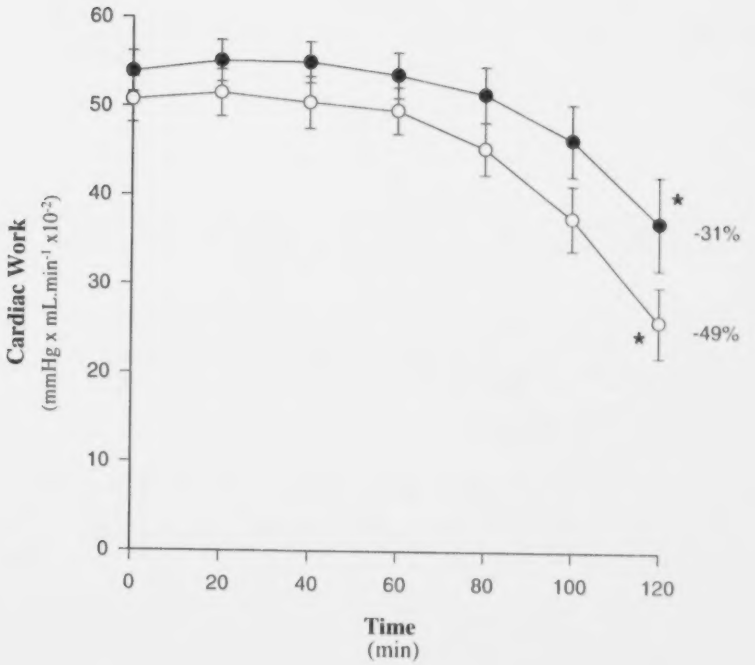


Figure 3.3 Effect of perfusate calcium concentration on cardiac work over time Two groups of hearts were perfused with either 1.25mM free Ca^{2+}/palmitate (○) or 2.5mM free Ca^{2+}/palmitate (●) for 2hr and cardiac mechanical function was measured as a function of cardiac work. Both the 1.25mM Ca^{2+}/0.8mM palmitate and the 2.5mM Ca^{2+}/0.8mM palmitate groups, showed significant loss of function at 2hr compared to t=0 hr within the same group (-49%, n=9; and -31%, n=9, respectively, $*p<0.05$ by Students' *t* test). Cardiac work was stable in both groups during the first 80 min. of perfusion. However, increasing calcium concentration in the perfusate was not able to prevent the loss of function seen over time in the isolated working rat heart.

4. *Effects of buffer calcium levels on cardiac response to preload changes over time*:

Figure 3.4 describes the response as cardiac work to changes in preload pressures (5.5-15.5 mmHg) in the two groups of hearts perfused with either low or high calcium levels in the perfusate at the beginning and at the end of the perfusion protocol. At t=0 hr, both groups showed an increase in cardiac work with increasing preload pressure. However, after 2hr perfusion, there was a significant loss in response to preload changes in the low calcium group. Whereas in contrast, the high calcium group showed an improvement in mechanical function compared to the low calcium group at t=2 hr and was significantly different at preload pressures of 13.5 and 15.5 mmHg ($p<0.05$).

5. *Effects of Cx on cardiac performance over time*:

Figure 3.5 shows the effects of Cx on cardiac function over time in hearts perfused with 1.25mM Ca^{2+}/0.8mM palmitate buffer in the presence of palmitate. In the absence of Cx, control hearts showed a 49% loss in mechanical function as cardiac work after 2hr perfusion. Addition of 10μM Cx to perfusate prevented the loss of function seen over 2h perfusion. At t=2hr, the mechanical function in the control group (was significantly different from the Cx group (**d** *vs.* **h**; $p<0.01$, Table 3.1). Cx prevented the loss of coronary flow over time (Table 3.2) in these hearts. At the beginning of the perfusion protocols, the mechanical functions in the two groups, control and Cx, were not statistically different from each other and, therefore, the two groups have similar starting conditions and were functionally comparable as shown in Table 3.1 (**c** *vs.* **g**).

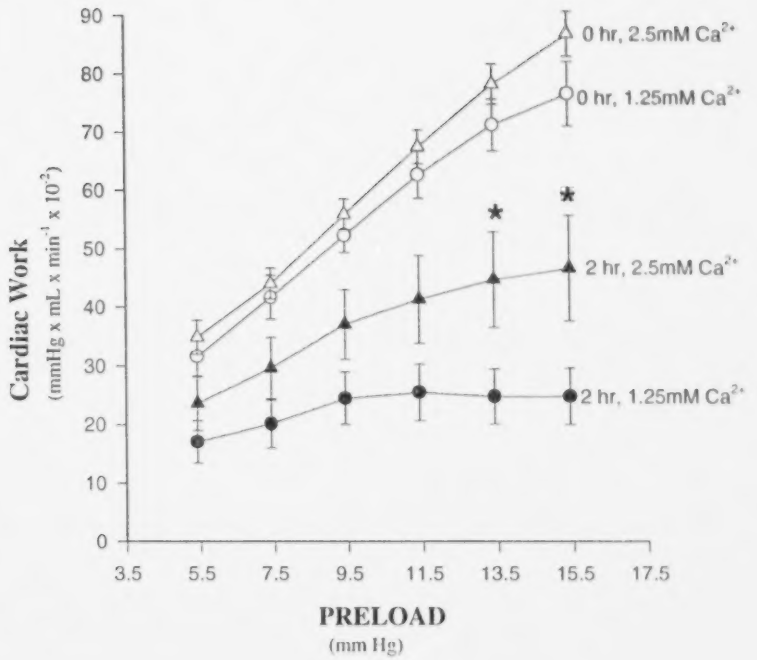


Figure 3.4 Preload pressure-cardiac work plot at 0hr and 2hr perfusion. Perfusate contains 0.8mM palmitate and either 1.25 or 2.5mM Ca^{2+}. At t=0 hr, both groups perfused with either 1.25mM Ca^{2+}/0.8mM palmitate (○) or 2.5mM Ca^{2+}/0.8mM palmitate (△) showed an increase in response given as cardiac work with increasing preload pressure from 5.5 to 15.5 mmHg. After 2hr of perfusion, the response to preload changes was significantly reduced in the lower calcium group at all preload pressures (●, $p<0.05$, *vs.* 0hr, n=9; Students' *t* test). This loss of function was reduced after 2hr of perfusion in the higher calcium group (▲) and was significantly greater than the lower calcium group at preload pressures of 13.5 and 15.5 mmHg at t=2 hr (*$p<0.05$, n=9; Students' *t* test). Therefore, increasing extracellular calcium concentration reduces, rather than prevents, the loss of function seen over 2hr of perfusion in the isolated working rat heart.

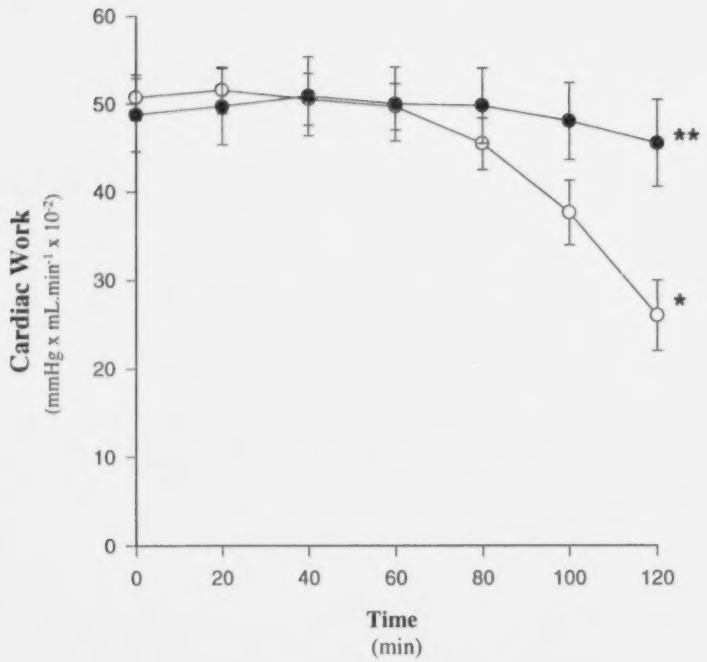


Figure 3.5 Effect of Cx on cardiac work over time in 1.25 mM Ca^{2+}/0.8mM palmitate perfused hearts. Hearts were divided into two groups and perfused with 1.25mM Ca^{2+}/palmitate buffer in the absence (Control) or presence of 10μM Cx (Cx) over 2hr. Cardiac function was measured as cardiac work. Control group (○) showed a significant loss of function at t=2 hr compared to starting conditions at t=0 hr (*$p<0.05$, n=9). Addition of Cx (●) to the perfusate prevented the loss of function following 2hr of perfusion and was significantly different from control group (**$p<0.01$, n=6). Therefore, loss of cardiac mechanical function over time occurred in hearts perfused with low calcium/palmitate buffer and was prevented by the protein synthesis inhibitor Cx.

6. *Effects of Cx on cardiac response to preload changes over time*:

Figure 3.6 shows the effects of Cx on the mechanical response of heart perfused with palmitate under low calcium conditions (1.25mM free Ca^{2+}). Both groups, control and Cx, showed a clear increase in response to increases in preload pressures (5.5-15.5 mmHg) at the beginning (t=0 hr) of perfusion. After 2hr, the control group showed a marked loss in mechanical response at all preload pressures tested ($p<0.01$, n=9; Students' *t* test). Addition of 10μM Cx to the perfusate prevented this loss in response compared to the control group, such that cardiac work was not significantly different at 2hr compared with 0hr values. Therefore, inhibition of *i*NOS protein synthesis in isolated working rat hearts preserved the Frank-Starling mechanism over 2hr of perfusion.

7. *NOS activity in hearts perfused under different buffer conditions* (Table 3.3):

Both Ca^{2+}-dependent and Ca^{2+}-independent NO synthase activity was determined by the citrulline assay in ventricular tissue cytosols of hearts perfused with 1.25mM Ca^{2+}/0.8mM palmitate (control, n=9), the group which showed the greatest loss in mechanical function over time, and compared to those perfused in the presence of Cx (n=6), the group which showed no loss in function over time. In both control and Cx groups the Ca^{2+}-dependent NOS activity was found and was not significantly different. However, iNOS activity was found in the control group, whereas no activity was found in Cx-treated hearts ($p<0.001$, Students' *t* test). Therefore, the enhanced iNOS activity seen in the control group could be contributing to the time-dependent loss of mechanical function in this group.

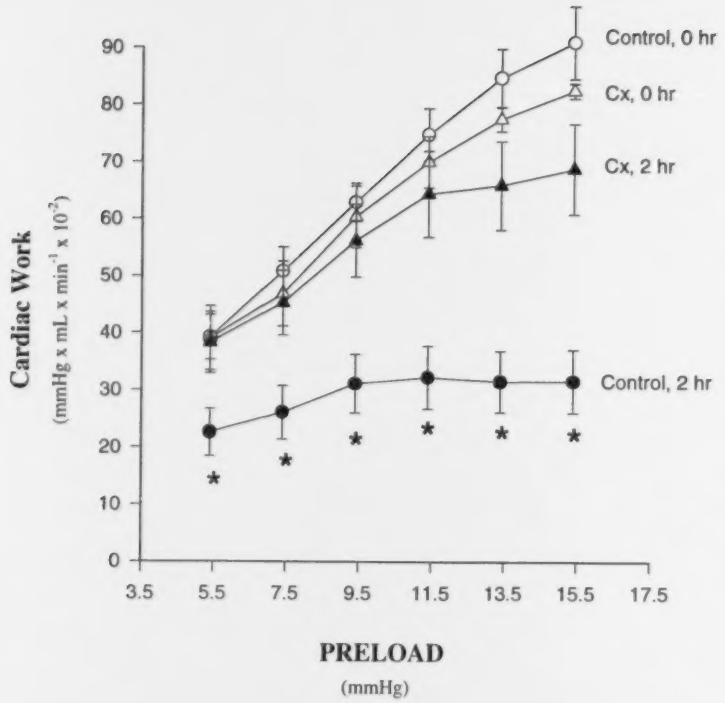


Figure 3.6 Preload pressure-cardiac work plot at 0hr and 2hr perfusion. Perfusate contains 1.25mM Ca^{2+} /0.8mM palmitate in the presence or absence of Cx. Two groups of hearts were perfused with 1.25mM Ca^{2+}/palmitate buffer in the absence (Control) or presence of 10μM cycloheximide (Cx). At t=0 hr, control (○) and Cx (△) groups showed an increase in response given as cardiac work with every increase in preload pressure from 5.5 to 15.5 mmHg. After 2hr of perfusion, the response to preload changes was significantly reduced in the control group (●) at all preload pressures ($p<0.05$, n=9). This loss of function was prevented in the Cx group (▲) after 2hr of perfusion and significantly different from the control group at all preload pressures measured at t=2 hr (*$p<0.05$, n=9). Therefore, Cx prevents the loss of response to preload changes seen over 2hr of perfusion in the isolated working rat heart.

Table 3.3 NOS activities in homogenates of hearts perfused over 2hr with 1.25mM free Ca^{2+}/0.8 mM palmitate in the absence(control) or presence of 10μM Cx. Ca^{2+}-dependent activity was seen in both groups and was not significantly different. Control group, however, showed an induction of Ca^{2+}-independent activity which was prevented in the Cx treated group ($p<0.001$ vs. control). Student's *t* test was used for statistical comparisons among the groups.

Perfused Hearts (t=2hr)	**NOS Activity** *(pmol/mg protein/min)*	
	Ca^{2+}-Dependent	**Ca^{2+}-Independent**
Control (n=9)	0.88 ± 0.19	0.60 ± 0.09
Cx (n=6)	0.90 ± 0.13	0.05 ± 0.04*

* *$p<0.001$* vs. Control hearts

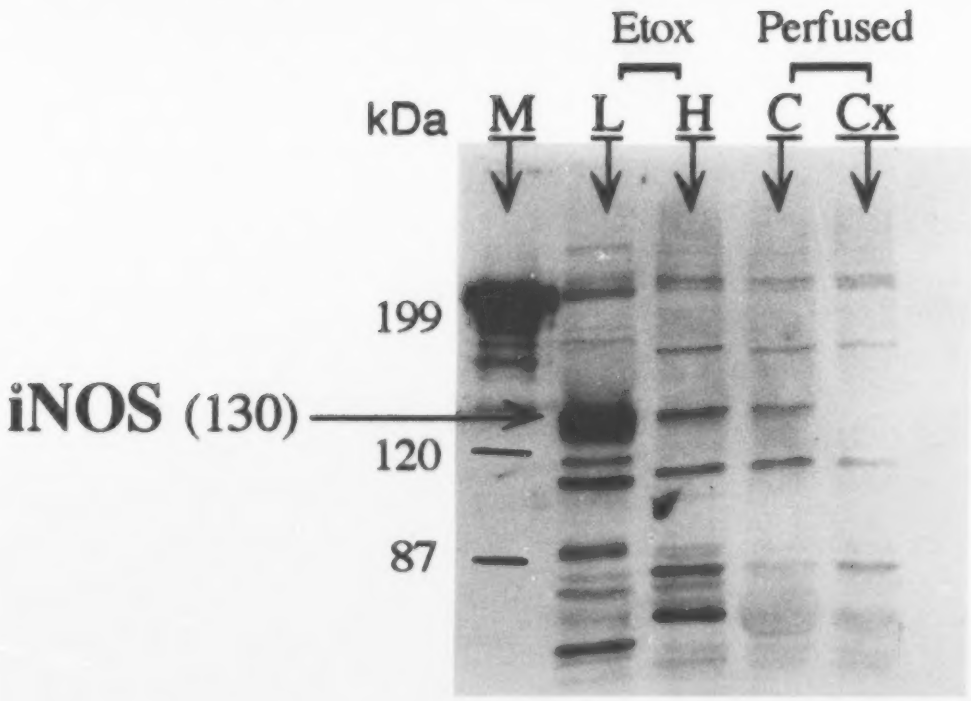
8. *Expression of iNOS in perfused hearts*: (Figure 3.7)

Western blot analysis were performed on cytosolic fractions from the ventricles of hearts perfused with 1.25mM Ca^{2+}/0.8mM palmitate buffer in the presence or absence of Cx. Samples of liver and hearts from rats injected with LPS for 6hr the time at which iNOS activity is maximal in the heart (Schulz *et al*., 1992), were used as positive controls for comparison. These samples showed expression of iNOS protein with a clear and intense bands of 130kDa molecular weight (lanes L and H). Control hearts perfused in the absence of Cx (lane C) showed a band at 130kDa verifying the expression of iNOS in these hearts. However, in the Cx treated hearts (lane Cx), there was no detectable expression of iNOS. Similar results were obtained with at least 2 hearts from each group (data not shown). Therefore, the protein synthesis inhibitor, Cx, prevented the expression of iNOS protein during 2hr of perfusion.

9. *Perfusate endotoxin levels* :

Measurements of endotoxin levels in perfusates using the E-toxate kit was performed in our laboratory by Ms. Donna Panas. These measurements show that the distilled water used to make perfusate had no detectable endotoxin, whereas the perfusate buffer contained 0.8ng/mL endotoxin before recirculation through the perfusion apparatus and 1.6ng/mL following 10min recirculation through the apparatus without a heart in the circuit. Perfusion for 2hr with an isolated working rat heart increased the level of endotoxin to four fold of its initial concentration in the perfusate.

Figure 3.7 Western blot analysis of iNOS protein expression in ventricles from hearts perfused over 2 hr with 1.25mM Ca^{2+} /0.8mM palmitate in the presence or absence of Cx. Ventricular tissue was lysed in protein sample buffer as described in methods. Equal amounts of denatured proteins (90μg) were loaded and separated on a 9% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoblotting was performed using rabbit immune serum containing antibodies against rat and mouse iNOS. Cytosolic fractions obtained from both liver and hearts from rats injected for 6hr with endotoxin (Etox) were loaded in lanes L and H respectively as positive controls. Molecular size markers in kilodaltons (kDa) are shown on the left lane (M); arrow indicates the position of the 130 kDa iNOS protein. Clear positive bands of iNOS proteins are seen expressed in Etox liver and hearts, as well as in control perfused hearts (lane C). However, perfused hearts treated with Cx did not show the expression of iNOS protein (lane Cx). These results were repeated in at least 2 hearts from each group with similar results.


Etox
Perfused
kDa
M
L
H
C
Cx
199
iNOS (130)
120
87

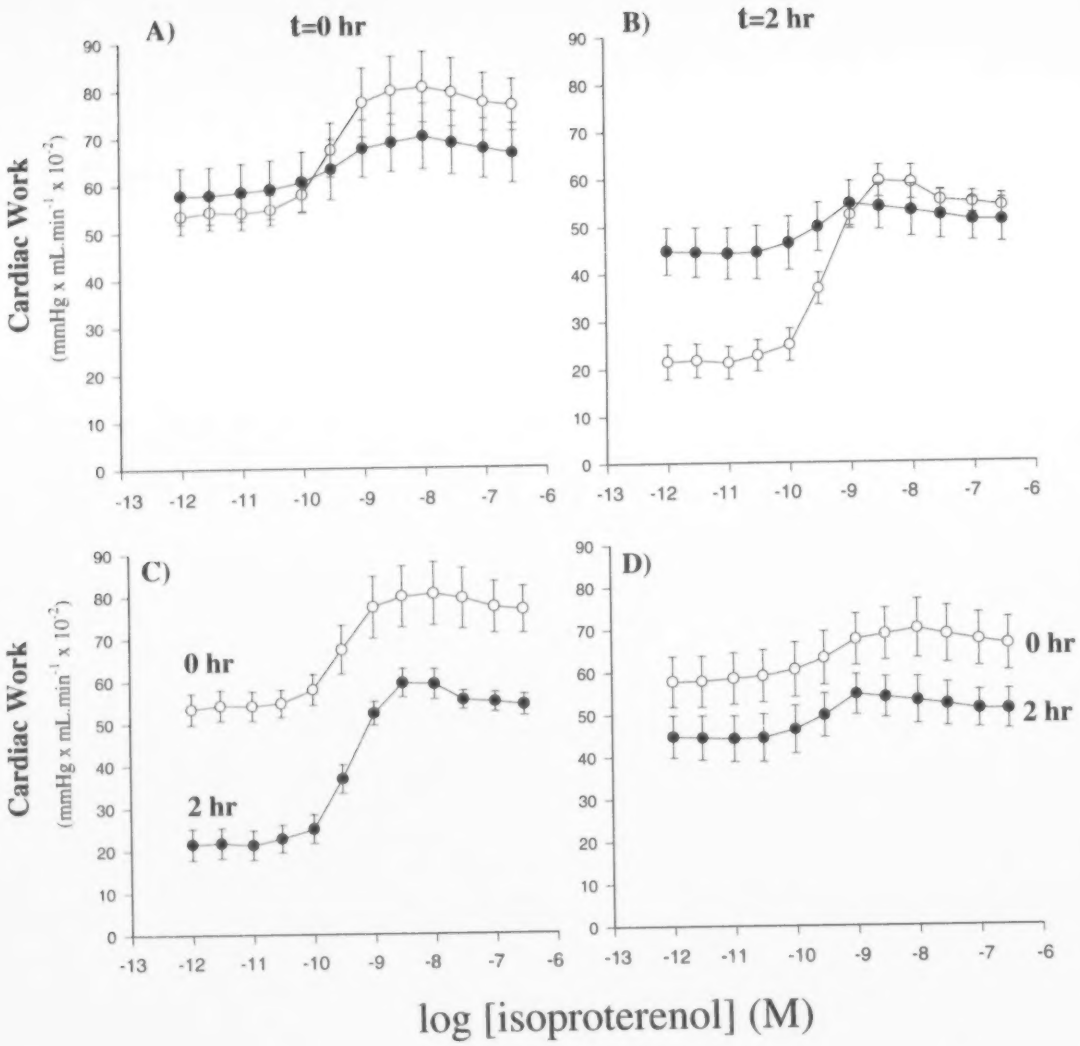
10. *Effects of Cx on dose response to isoproterenol in hearts at beginning and at the end of perfusion protocol*:

Figure 3.8 shows the dose response to the β-agonist, isoproterenol, in hearts perfused with 1.25mM Ca^{2+}/0.8mM palmitate in the absence or presence of 10μM Cx at t=0 hr (Figure 3.8A) and after 2hr (Figure 3.8B) of *in vitro* perfusion. Addition of Cx to the perfusate did not show any significant difference in the maximum response at t=0 hr, which was obtained at 10nM isoproterenol concentration, compared to the control group at the beginning or after 2hr of perfusion. Responses to cumulative doses of isoproterenol were significantly different ($p<0.05$) in the control group at t=0 hr compared to t=2 hr within the same group, achieving a maximum response at t=0 hr at the isoproterenol concentration of 10nM. In this group, the maximum response was higher at t=0 hr than that tested after 2hr of perfusion. However, after 2hr of perfusion Cx was able to correct the extent of functional loss at the same concentration which achieved maximal response in control hearts perfused in the absence of Cx. Calculation of the mean effective concentration of isoproterenol at 50% of maximal response (EC_{50}) in each group did not show any significant difference between the two groups (Table 3.4).

11. *Role of buffer calcium in dose-response to isoproterenol of hearts following 2hr of perfusion:*

The response to cumulative doses of the β-agonist, isoproterenol, was tested in isolated working rat hearts following 2 hr of perfusion with 0.8mM palmitate

Figure 3.8 Concentration-dependent increase in cardiac work to isoproterenol in hearts perfused with or without Cx in 1.25mM Ca^{2+} /0.8mM palmitate buffer at t=0 hr or after 2 hr perfusion. Panels A and B show comparisons between dose-responses of both Ca^{2+}/0.8mM palmitate (control, ○) and Cx (●) groups at t=0hr and t=2hr respectively. Panel C shows the response as cardiac work of control group of hearts to cumulative concentrations of isoproterenol at 0hr and after 2hr perfusion. The maximal response to isoproterenol was reduced significantly in the control group at t=2hr of perfusion. Panel D shows response of hearts perfused with 1.25mM Ca^{2+}/0.8mM palmitate buffer, in the presence of 10μM Cx, to cumulative doses of isoproterenol at 0hr and after 2hr perfusion. There was no significant time-dependent change in response to isoproterenol in Cx-treated group. Addition of Cx did not show any significant effects on the maximum response to isoproterenol compared to control group at t=0 hr. This maximum response was achieved at 10nM concentration in both, control and Cx-treated groups. At t=2hr, maximum response was achieved with 3nM isoproterenol in control group but it was lower than that obtained in groups tested at t=0hr. The maximum response to isoproterenol achieved by Cx-treated hearts was similar to that obtained by control hearts at t=2hr. Mean EC_{50} value in Cx group was not significantly different from that of control group at t=2hr. At maximum doses, isoproterenol corrects the extent of cardiac function loss observed at t=2 hr in control hearts.


A)
t=0 hr
B)
t=2 hr
C)
D)
0 hr
2 hr
Cardiac Work
(mmHg x mL.min^{-1} x 10^{-2})
log [isoproterenol] (M)

containing buffer under either 1.25mM Ca^{2+} (low) or 2.5mM Ca^{2+} (high) conditions (Figure 3.9). Responses to isoproterenol were expressed as cardiac work using cumulative doses from 10^{-12} M to 10^{-7} M. Hearts perfused with either low or high buffer calcium achieved similar maximum responses to isoproterenol at 3nM concentration after 2hr of perfusion. This maximum response obtained in the high calcium group to 3nM isoproterenol was not higher than that seen in low calcium perfused hearts at the beginning of the perfusion protocol. Estimation of the mean EC_{50} in either low or high calcium groups showed no significant difference analyzed by Students' *t* test (Table 3.4).

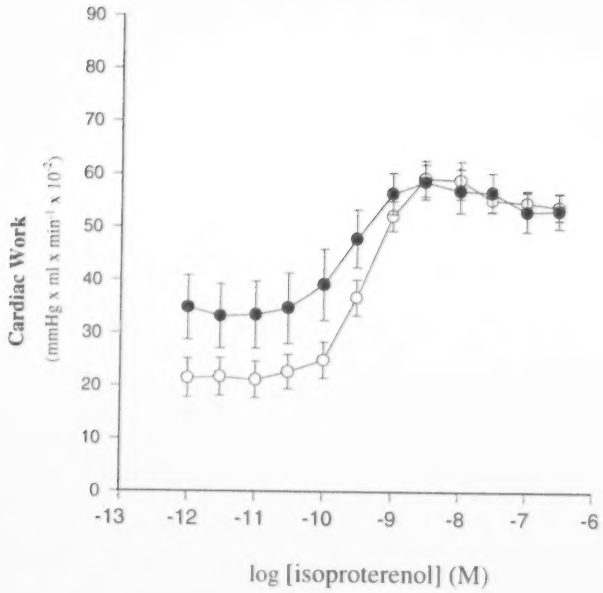


Figure 3.9 Concentration-dependent increase in cardiac work to isoproterenol in hearts perfused with 2.5mM Ca^{2+}/0.8mM palmitate buffer following 2hr of perfusion Response to isoproterenol is expressed as cardiac work and measured under constant workload conditions. Hearts perfused with either 1.25mM Ca^{2+}/0.8mM palmitate (○) or 2.5mM Ca^{2+}/0.8mM palmitate (●) buffers achieved similar maximum responses to isoproterenol at 3nM concentration. Estimation of the mean EC_{50} in either 1.25 or 2.5mM calcium perfused groups showed no significant difference (Table 3.4).

Table 3.4 Values of EC_{50} for isoproterenol on stimulation of cardiac work in hearts perfused with different buffer conditions at either t=0hr or t=2hr. EC_{50} is expressed as the mean ± SEM (nM) and showed no significant difference among all groups studied using Student's unpaired *t* test. "n" indicates the number of hearts studied in each group. The values for EC_{50} were calculated using computer-assisted estimation using GraphPad Inplot software (GraphPad Inc.)

Perfusion Condition	**EC_{50}** (nM)	**n**
1.25mM Ca^{2+},0.8mM palmitate/t=0hr	0.27 ± 0.05	6
1.25mM Ca^{2+},0.8mM palmitate/t=2hr	0.35 ± 0.05	9
2.5mM Ca^{2+},0.8mM palmitate/t=2hr	0.21 ± 0.08	9
1.25mM Ca^{2+},0.8mM palmitate,10μM Cx/t=0hr	0.26 ± 0.12	6
1.25mM Ca^{2+},0.8mM palmitate,10μM Cx/t=2hr	0.20 ± 0.13	6

CHAPTER IV

DISCUSSION

In this study, the mechanical function of isolated working rat hearts has been investigated using various buffer conditions and tools to determine the possible cause of loss of function over time. The working heart model was used in this study because it permits the gathering of a great deal of information under highly controlled conditions. Therefore, estimation of certain aspects of cardiac muscle mechanics, and of the function of the heart as a pump was possible in using this model. Since the first model of isolated working heart was introduced by Neely *et al.* (1967), it has been later improved by others who used this model to study substrate utilization in relation to work performance over time (Scheuer 1977; Taegtmeyer *et al.*, 1980; Lopaschuk *et al.*, 1990). Perfusion of isolated working hearts over a 2hr period shows a depression in contractile mechanical function, measured as cardiac work, which is delayed in onset (Schulz *et al.*, 1995). It has been shown that the isolated working rat heart loses about 20% of its cardiac output by 2hr of perfusion (Taegtmeyer *et al.*, 1980). The mechanism by which this occurs *in vitro* is not well understood and may be partially dependent upon workload, extracellular calcium concentration, temperature, as well as the energy substrate provided for supporting mechanical work (Taegtmeyer *et al.*, 1980).

In the working heart a corresponding high rate of mitochondrial production of ATP is required because of the very high turnover rate of ATP in the myocardium. Pyruvate was used as an energy substrate, in addition to glucose that is already present in the perfusate. Insulin (100μU/mL) was added to the buffer in order to enhance glucose utilization by the heart. The first group of hearts were perfused with 1.25mM Ca^{2+}/5mM pyruvate buffer for 2 hr. In this group, mechanical function was stable during the first 80 minutes of the perfusion protocol after which the drop in function, as cardiac work, was significantly pronounced at the

end of the perfusion period at t=2 hr with a 24% decrease in cardiac work. The major pathways of pyruvate metabolism are either aerobic oxidation via pyruvate dehydrogenase and the citrate cycle, or anaerobic conversion to lactate (Randle, 1976). Aerobic oxidation of pyruvate requires first the activity of pyruvate dehydrogenase which can be enhanced up to 60-90% by the provision of pyruvate from increased glycolytic flux (Randle, 1976). In our preparation 5mM pyruvate has been added to the perfusate to enhance ATP formation and improve cardiac mechanical function in the isolated working heart. Thus, the presence of pyruvate as an energy substrate in the perfusate was not an ultimate tool in eliminating the role of other existing factors in the buffer.

Evans (1914) suggested that only one third of the heart's energy requirement is supplied by carbohydrates. However, the rest of the heart's energy requirements is obtained from fatty acids (Cruickshank *et al.*, 1941). In the absence of fatty acids, the isolated working heart will be depleted of its endogenous triglycerides over time (Saddik and Lopaschuk, 1991). Although triglycerides are not normally a major energy source for heart muscle, even after a high lipid meal in the working rat heart triglycerides can provide about 11% of its energy requirement and will be depleted if fatty acids are not provided as an energy substrate (Saddik and Lopaschuk, 1991) Therefore it is plausible that the loss in cardiac work over 2hr may be due to the lack of fatty acid in the perfusate. Therefore, pyruvate was replaced by the fatty acid, palmitate, which could be utilized by the heart as a high energy fuel source. Perfusion of hearts with 0.8mM palmitate in the perfusate did not reduce the loss of function over 2hr seen in the pyruvate perfused group. After 2hr of perfusion, the palmitate perfused hearts showed a significant loss of mechanical function compared to their baseline at t=0hr (49%) which, however, was not statistically significant compared to the

pyruvate group at t=2hr. The mechanical function was also stable during the first 80 minutes of perfusion. The response of hearts to changes in preload pressures was also studied at the beginning and after 2hr of perfusion using either pyruvate or palmitate in perfusate. At t=0 hr both pyruvate and palmitate-perfused groups showed an increase in mechanical function with each increase in preload pressure, suggesting an intact status of the Frank-Starling mechanism. However, by 2hr period this response was significantly reduced in palmitate perfused hearts at all preload pressures, indicating alterations in the Frank-Starling mechanism. This loss of function had a tendency to be lower, however, was not significantly different in the pyruvate perfused group at t=2hr compared to its original starting conditions. The presence of fatty acid should ensure the availability of large supply of ATP in order to sustain mechanical function over time, considering that utilization of ATP by the cardiac myocytes is efficient. However, the presence of other crucial factors could be involved in negating the beneficial action of palmitate in supplying ATP. It is necessary to mention that 3% BSA was used as a carrier for palmitate in the perfusate. This amount of BSA, which is not certified to be endotoxin free, likely accounts for some of the endotoxin that is present in the perfusion buffer. In the absence of palmitate, 0.2% BSA was only added to the buffer in order to provide a better surface wetting action of the buffer within the perfusion apparatus. Thus, the lack of fatty acid in the perfusate could not explain the loss of function observed in isolated working rat hearts. Therefore, the next suspected factor in this investigation is the availability of Ca^{2+} ions for the contractile machinery in cardiac cells.

Calcium plays a key role in regulating cardiac mechanical function. The interaction between the contractile proteins is tightly controlled by the cytosolic calcium ion concentration (Opie, 1995). In this case, increasing extracellular Ca^{2+}

concentration should enhance Ca^{2+} handling by the cardiac myocytes and prevent the loss of function over time. Another group of hearts were perfused with 2.5mM Ca^{2+} Krebs-Henseleit perfusate in the presence of 0.8mM palmitate. After 2hr of perfusion, this group showed significant loss of function (31%) compared to starting conditions at t=0hr within the same group. However, this doubling of the extracellular Ca^{2+} concentration reduced but did not prevent the loss of function seen in the 1.25mM Ca^{2+} /0.8mM palmitate perfused group. There was no significant difference in mechanical function at t=2hr between the two groups. Preload function studies at t=2 hr in the 2.5mM calcium perfused hearts revealed that increasing buffer Ca^{2+} concentration enhanced the response of these hearts to changes in preload pressures and showed a significant improvement over the lower calcium perfused group at high preload pressures. The loss of function observed in our preparations led to a question of whether NO may be involved in the loss of function over 2hr of *in vitro* perfusion. In this study, the possibility of involvement of other factors in function loss over time was also studied.

Pretreatment of hearts with the protein synthesis inhibitor Cx in 1.25mM Ca^{2+}/0.8mM palmitate buffer prevented the loss of function over time, suggesting that *de novo* protein synthesis was necessary for the development of cardiac depression. These results are in agreement to those of Schulz *et al.* (1995) in hearts perfused with 1.25mM Ca^{2+}/5mM pyruvate where the loss in function in either control or interleukin-1β and tumor necrosis factor-α treated hearts was abolished by Cx pretreatment. Responses to changes in preload pressures were also preserved in Cx-treated hearts. Measuring NOS activities in control hearts perfused for 2hr with 1.25mM Ca^{2+} /0.8mM palmitate buffer indicated significant

iNOS activity. Cx prevented the expression of iNOS activity which could be involved in cardiac depression over time. To verify the expression of iNOS protein, Western blot analysis was performed on cytosolic fractions from either control or Cx-treated hearts and revealed the existence of a 130 kDa band indicative of iNOS protein expression in control untreated hearts whereas it was not present in the Cx treated group. Therefore, the expression of iNOS was associated with the loss of function seen in the 1.25mM Ca^{2+}/0.8mM palmitate perfused group and could be due to the inadvertent exposure of hearts to bacterial endotoxin during the perfusion itself, or may be due to a spontaneous release of cytokines in the isolated heart. Endotoxin was found in detectable levels higher than 1ng/mL in the pyruvate-containing perfusate, and suggesting that BSA may be the major source of endotoxin, one could predict that levels of endotoxin may have been higher in the palmitate-perfused hearts. This level of endotoxin is enough to trigger the induction of iNOS which may be contributing to the loss of function over time mediated through excess of NO production under these conditions (Rees *et al.*, 1990; Schulz *et al*, 1995). Indeed we need to test whether addition of 3% BSA to 5mM pyruvate buffer would give same effect as 3% BSA in 0.8mM palmitate buffer.

The positive inotropic response of the isolated working hearts to the β-agonist, isoproterenol, was tested to demonstrate any functional changes in the β-adrenergic receptor activity after 2hr of perfusion. At t=2hr, the 1.25mM Ca^{2+}/0.8mM palmitate group showed a diminished response at all concentrations of isoproterenol than that of the same group at t=0hr. This indicates that the capacity of the cardiac myocytes to respond to β-adrenergic stimulation was reduced after 2hr of perfusion. At t=2 hr, hearts perfused with either 1.25mM

Ca^{2+} or 2.5mM Ca^{2+}/0.8mM palmitate buffer obtained similar maximum response to isoproterenol at a concentration of 3nM. Addition of Cx did not show any significant difference in the maximum response at t=2hr which was achieved at 1nM isoproterenol concentration. The mean EC_{50} in each group was not significantly different amongst all groups, suggesting no change in β-adrenergic sensitivity to isoproterenol over time. However, isoproterenol was able to correct the extent of functional loss observed at t=2hr at doses that gave maximal responses in the 1.25mM Ca^{2+}/0.8mM palmitate perfused buffer at t=2hr of perfusion. This indicates a compensatory role of a positive inotrope in enhancing cardiac function during early stages of cardiac failure. A selective depression in β-adrenergic responsiveness occurs in cardiac myocytes following cytokine treatment (Lange and Schreiner, 1992) which is reversible with the NOS inhibitor L-NMMA (Ungureanu-Longrois *et al.*, 1995b). It is not clear yet whether uncoupling of the β-adrenergic receptor with adenylate cyclase occurred in NO-mediated hyporesponsiveness to β-agonists or whether the increase in rate of degradation of cAMP is involved via elevation of cGMP upon the stimulation of guanylate cyclase by enhanced NO levels (Goldberg *et al.*, 1975; Lohmann *et al.*, 1991b). Levels of cAMP in heart tissues were often found to be reduced when cGMP levels were increased, and biochemical evidence exists for the presence of a cGMP-stimulated cAMP-phosphodiesterase which degrades cAMP in cardiac tissues (Lohmann *et al.*, 1991b; Watanabe and Besch, 1975).

Coronary flow was monitored in all groups studied and varied within a small range (20-25 mL/min) (Table 3.2). However, there was a significant decrease ($p<0.01$, n=9) in coronary flow measured at t=2hr of perfusion in 1.25mM Ca^{2+}/0.8mM palmitate perfused hearts. Cx prevented this reduction in coronary flow in 1.25mM Ca^{2+}/0.8mM palmitate perfused hearts. Other groups did not

show any significant changes in coronary flow over 2hr of perfusion (Table 3.2).

Expression of iNOS in cardiac tissues contributes to the enhanced formation of NO which mediates cardiac depression by either the stimulation of guanylate cyclase (Smith *et al.*, 1991; Shah and Lewis, 1993; Brady *et al.*, 1993) or by direct inhibitory actions of NO on mitochondrial respiratory enzymes (Drapier and Hibbs, 1988; Geng *et al.*, 1992) or through other unknown actions that needs further investigations.

CONCLUSIONS: It can be concluded that the loss of cardiac function over time in the isolated working rat heart:

a) cannot be due to the lack of fatty acid in the perfusate, a major energy substrate in the heart,

b) is reduced in magnitude by increasing perfusate Ca^{2+} concentration

c) may be abolished by Cx, which may be acting by preventing an increase in iNOS expression and activity in the heart, and

d) could be corrected by a positive inotrope isoproterenol, suggesting that an irreversible defect in cardiac function had not occurred by this time

CHAPTER V

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